Rat Brain Chloride Intracellular Channel Protein CLIC4: Subcellular Localisation and Protein Interaction Studies

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DECLARATION

The work reported in this thesis was carried out under the supervision of Dr. Richard H Ashley at the Section of Biomedical Sciences, Division of Biomedical and Clinical Laboratory Sciences, University of Edinburgh. All results presented, unless otherwise stated, are the sole work of the author, as is the composition of this thesis.

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Abbreviation / Symbol	Term
A	absorbance
Ab	antibody
AEBSF	4-(2-aminoethyl)-benzenesulphonyl fluoride
bp	base-pair(s)
BSA	bovine serum albumin
C	carboxy
Ca^{2+}	calcium
cAMP	cyclic adenosine 5'-phosphate
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane regulator
CK II	casein kinase II
Cl^-	chloride
CLIC	chloride intracellular channel
Cyt. D.	cytoplasmic domain
°C	degrees Celsius
D	dalton
DIDS	4,4'-diisothiocyanostilbene-2, 2'-disulfonic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNDS	4,4'-dinitrostilbene-2-2'-disulphonic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	dithiothreitol
ECL	enhanced-chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(-aminoethylether) , , '-tetraacetic acid
ER	endoplasmic reticulum
FBS	foetal bovine serum
g	gravity
GABA	-amino-butyric acid receptors
hr	hour(s)
HRP	horseradish peroxidase
IAA	indanyloxyacetic acid
IgG	immunoglobulin G

IPTG	isopropyl- -thiogalactopyranoside
IP_3	inositol 1,4,5-trisphosphate
IP_3R	inositol 1,4,5-trisphosphate receptor
K^+	potassium
kb	kilobase-pair(s)
kDa	kilodalton
LGICs	ligand-gated ion channels
M	molar
MES	2-[N-morpholino] ethanesulphonic acid
Mg^{2+}	magnesium
mg	milligram
min	minute(s)
ml	millitre
mM	millimolar
mm	millimeter
MOPS	4-morpholinepropane sulphonic acid
µCi	microCurie
µg	microgram
µl	microlitre
µm	micron
µM	micromolar
N	amino
ng	nanogram
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFU	plaque-forming unit(s)
P_i	orthophosphate
pmoles	picomoles
PKA	cAMP-dependant protein kinase
PKC	protein kinase C
PLM	phospholemman
POPE	1-palmitoyl-2-Oleoyl-sn-Glycero-3-Phoshpoethanolamine
POPS	1-palmitoyl-2-Oleoyl-sn-Glycero-3-[Phospho-L-Serine)
rATP	adenosine 5'-triphosphate
RNA	ribonucleic acid

rpm	revolutions per minute
RyR	ryanodine receptor
sec	second(s)
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SR	sarcoplasmic reticulum
TBE	Tris-borate-EDTA buffer
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetramethylenediamine
Tm	transmembrane domain
Tm	melting temperature
Tris	tris- [hydroxymethyl]-aminomethane
Triton X-100	octylphenyl-nonaoxyethylene
W	Watt(s)
w/v	weight per volume
w/w	weight per weight

ABSTRACT

Rat brain CLIC4 (p64H1) is a member of a family of recently identified chloride intracellular channel (CLIC) proteins that share homology with the putative bovine anion channel protein p64. CLIC4 comprises a 28 kDa protein, with a large cytoplasmic domain, a single transmembrane domain and a small intraluminal domain. Similar to other CLIC proteins, CLIC4 shows a wide tissue distribution with an unusual dual localisation in both membrane and cytoplasmic fractions. However, the molecular bases of its cellular localisation, and anion channel activity are unclear. To help address these problems, this study set out to identify CLIC4-associated proteins. Recombinant full-length and truncated CLIC4 were expressed in bacteria as glutathione-S-transferase (GST) fusion proteins, and purified by glutathione affinity chromatography. In "pull-down" assays, several rat brain cytosol proteins were found to bind directly or indirectly to the cytoplasmic domain of CLIC4, including dynamin I, -actin, tubulin and 14-3-3 isoforms. These interactions were confirmed *in vivo* by immunoprecipitation. CLIC4 was over-expressed in cultured mammalian cells as full-length and Flag-tagged fusion proteins. The subcellular localisation of native and heterologously expressed CLIC4 was investigated by indirect immunofluorescence. Biochemical and immunofluorescence analyses of CLIC4-transfected cells demonstrated partial co-localisation of CLIC4 with caveolin, and with functional caveolae. Several consensus phosphorylation sites have previously been identified in the CLIC4 protein sequence. CLIC4 interactions with protein kinases were confirmed by demonstrating phosphorylation by protein kinase C, protein kinase A and tyrosine kinase *in vitro*. The incorporation of purified recombinant CLIC4 into planar lipid bilayers failed to demonstrate specific anion channel activity, suggesting that interactions with some of the identified cellular proteins might underlie the anion channel activity of CLIC4.

CHAPTER 1

INTRODUCTION

1.1 ION CHANNELS

The membrane that surrounds all cells contains a distinct array of receptors, transporters and transmembrane channels that are characterised by a great structural and functional diversity. Amongst all the proteins available for study, it is the membrane ion channels that have been the most enigmatic to investigate. Ion channels are macromolecular protein tunnels that span the membrane lipid bilayer. They facilitate ion movement across phospholipid membranes by forming through the membrane an aqueous pore, which is lined by polar groups. The pore does not determine the direction of ion flow, but only its rate. Due to the size of the pore, ions move through the channel in single file and must partially dehydrate. Interactions of the ion with the polar groups in the pore replace the energy lost by dehydration. An infinitesimally small amount of ions need to pass through the membrane lipid bilayer to generate a large membrane potential. Moreover, the turnover rate of an ion channel can be as high as 10^8 /sec, explaining the low abundance of channel proteins in membranes.

The isolation of the ion channels becomes more complicated due to the hydrophobicity of the proteins, making them difficult to purify. An additional problem is that many different channels have been identified within a single cell. Despite the problems arising from the purification of channel proteins, a plethora of ion channels have been purified, cloned and reconstituted by a variety of techniques. Ion channels are distinguished by their ion specificities, the time courses of their activation and inactivation, as well as the stimuli that regulate them.

This chapter focuses on Cl^- channels present in cell-plasma membrane and intracellular organelles. Intracellular calcium and potassium channel proteins are also briefly discussed.

1.2 PLASMA MEMBRANE CHLORIDE CHANNELS

Chloride (Cl^-) is the most abundant extracellular anion in multicellular organisms. Cl^- channels facilitate the passive movement of Cl^- into and out of cells as well as between the intracellular compartments. A plethora of distinct Cl^- channel activities have been described with a variety of essential roles in cellular physiology. Cl^- channels in the plasma membrane play important roles in cell volume regulation so that cells are able to adapt to changing tonicity; in transepithelial transport of salt and water by epithelial tissues such as in the kidney, and in setting the membrane potential providing electrical stability in excitable tissues such as skeletal muscle and neurons (reviewed by Jentsch, 1996; Jentsch and Gunther, 1997; Foskett, 1998).

To date, four structurally distinct and unrelated families of Cl^- channel proteins have been identified in plasma membranes: the ligand-gated family (γ-aminobutyric acid A - $GABA_A$ and glycine receptors; Hosie *et al.*, 1997), the cystic fibrosis transmembrane protein regulator (CFTR; Riordan *et al.*, 1989; Welsh, 1994), the Chloride Channel (ClC) family (Jentsch *et al.*, 1995) and the Ca^{2+}-activated Cl^- channel (CaCC) family (Fuller and Benos, 2000). CFTR and ligand-gated families have been studied intensively over the past several years, due to their molecular definition and roles in diseases.

1.2.1 LIGAND-GATED CHLORIDE CHANNELS

The first class to be discovered included the glycine (GlyR) and γ-amino-butyric acid A ($GABA_A$) receptors known to mediate fast synaptic inhibition in the mammalian brain (Grenningloh *et al.,* 1987; Schofield *et al.,* 1987; MacDonald *et al.,* 1994). The inhibitory GlyR and $GABA_A$ belong to the ligand-gated ion channel (LGIC) family, which also comprise the excitatory nicotinic acetylcholine receptor, nAChR (which form cation channels), serotonin type 3 (5'-HT_3) receptor, as well as the inhibitory glutamate receptors (Betz, 1990; Ortells and Lunt, 1995; Cleland, 1996). Ligand gated ion channels are generally formed as homo- or hetero-oligomeric assemblies of integral membrane protein subunits. Based on their homology to the nAChR receptor, LGICs are believed to be pentameric proteins, each subunit having a large extracellular domain at the *N*-terminal domain, four transmembrane segments (M1-M4), and an intracellular (M3-M4) loop (Figure 1-1A). Although the exact folding of LGIC proteins remains unknown, it has been suggested that five M2 domains, predominantly in the α-helical conformation, contribute to the central pore (Changeux *et al.*, 1992; Galzi and Changeux, 1995; Akabas and Karlin, 1995). $GABA_A$ receptors are more complex and are believed to form hetero-oligomers composed of subunits from six classes with several isoform (α1-6, β1-3, γ1-3, δ, ε, θ, π). The major receptor isoforms is likely to be composed of α1, β2 and γ2 subunits (reviewed by Bauman *et al.,* 2001). In contrast, $GABA_B$ receptors are seven transmembrane domain proteins (Kaupmann *et al.*, 1997) that couple to either K^+ or Ca^{2+} channels via G proteins, and are regulated by intracellular second messenger systems (Bowery, 1989).

Recently, a third class of GABA receptor has been discovered, termed $GABA_C$. It was originally described in spinal cord interneurons (Johnston *et al.*, 1975; Drew *et al.*, 1984) and has been extensively characterised in the retina (Feigenspan *et al.*, 1993). $GABA_C$ receptors are composed of a novel class of GABA receptor subunits, termed ρ, which are able to form functional homo-oligomeric as well as hetero-oligomeric receptors (Greka *et al.,* 1998). Unlike $GABA_A$ receptors, $GABA_C$ receptors gate Cl^- currents with distinct electrical properties such as higher sensitivity for GABA, smaller current and inability to desensitise (reviewed by Enz, 2001).

Finally, the ligand-gated channel family also includes glutamate receptors (Wadiche *et al.*, 1995; Fairman *et al.*, 1995). Based on the structure and mechanisms of their action, glutamate receptors are categorised into two main groups, the ionotropic receptors and the metabotropic receptors. The ionotropic receptors are ligand-gated ion channels that mediate fast synaptic transmission whereas the metabotropic receptors are coupled to G proteins and initiate signalling cascades. Three distinct subfamilies of ionotropic receptors have been identified and characterised by their affinity for their agonists, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; Hollmann *et al.*, 1989; Keinanen *et al.*, 1990; Boulter *et al.*, 1990), NMDA (*N*-methyl -D-aspartate; Moriyoshi *et al.*, 1991; Meguro *et al.*, 1992; Ishii *et al.*, 1993) and kainate (Egebjerg *et al.*, 1991; Werner *et al.*, 1991; Bettler *et al.*, 1992). Upon activation AMPA and kainate receptors are responsible for primary synaptic signalling events. Kainate receptors are also involved in the modulation of the presynaptic release of neurotransmitter (Bettler *et al.*, 1992). NMDA receptors become fully activated only when blocking by extracellular Mg^{2+}

is relieved by depolarisation of the membrane (Nowak *et al.*, 1984). The metabotropic glutamate receptor family includes eight different subtypes, categorised into three main groups based on their sequence homology (reviewed by Cartmell and Schoepp, 2000). Group I metabotropic glutamate receptors are usually found postsynaptically and their activation leads to slow depolarisation. Group II and III are generally found presynaptically where they modulate the release of glutamate or other neurotransmitters.

1.2.2 CYSTIC FIBROSIS TRANSMEMBRANE REGULATOR - CFTR

Cystic fibrosis (CF) is an autosomal recessive disorder, characterised by disturbances in ion secretion and the absorption of electrolytes. The symptoms of CF include thick airway mucus, pancreatic insufficiency, elevated sweat [Cl^-], intestinal blockage and chronic sinusitis (Collins, 1992). In an attempt to isolate the gene or genes mutated in CF, the cystic fibrosis transmembrane conductance regulator (CFTR) gene was discovered (Rommens *et al.*, 1989; Riordan *et al.*, 1989). Functional characterisation of CFTR showed that it is a cAMP regulated Cl^- channel (Rommens *et al.*, 1989) located in the apical membrane of several Cl^- secretory epithelia (Crawford *et al.,* 1991), and that it is a member of the ATPase-binding cassette (ABC) transporter family (Anderson and Welsh, 1992; Fuller and Benos, 1992). Electrophysiological studies of CFTR expressed in heterologous system indicated that unlike other members of the ABC family, CFTR can conduct Cl^- ions (conductance of ~10 pS), and it has a halide selectivity profile of $Br^- > Cl^- > I > F^-$ (Bear *et al.,* 1991; Anderson and Welsh, 1992).

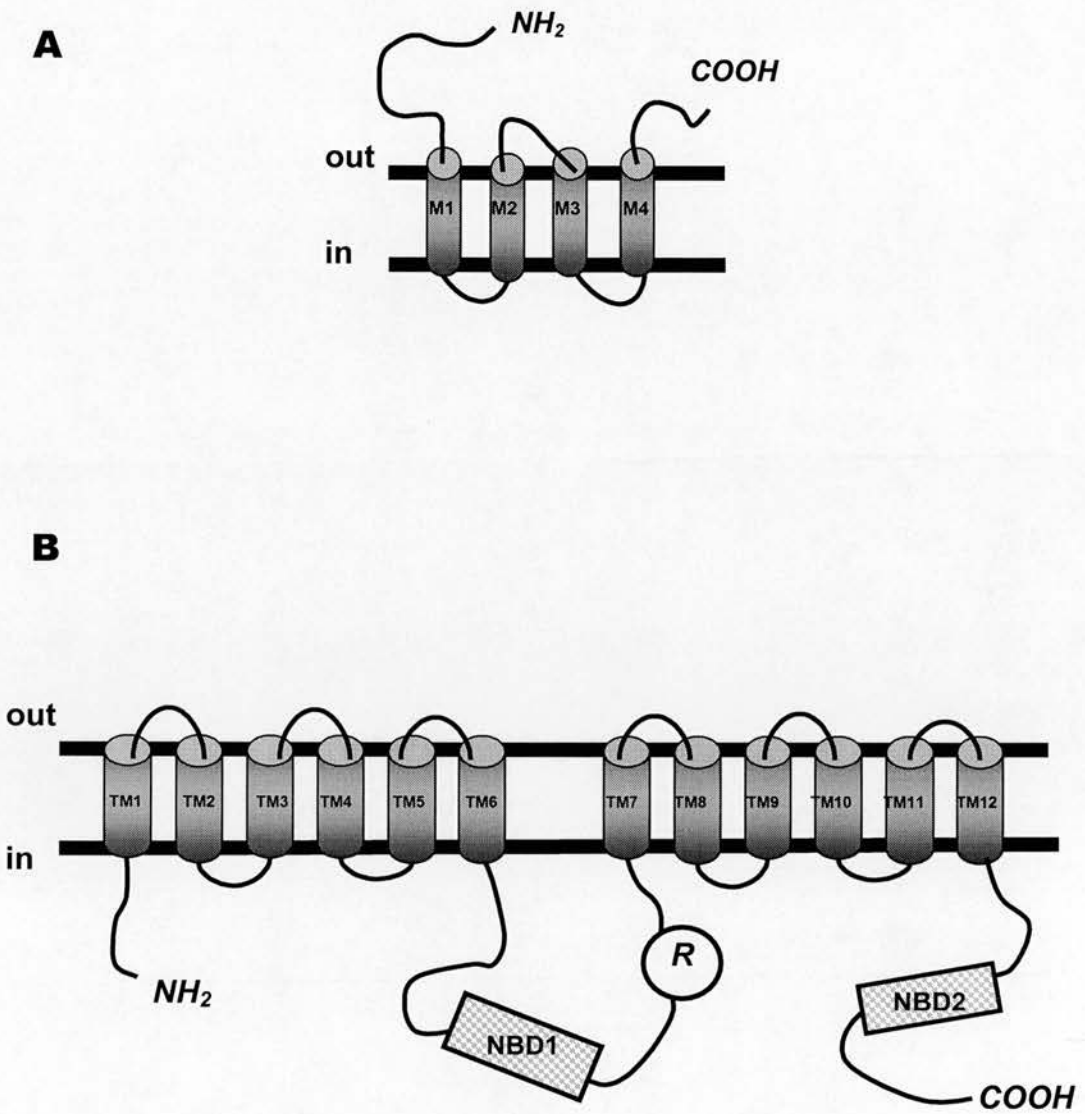


Figure 1-1. Proposed topologies of LGIC and CFTR channels.

A. Membrane topology model for the ligand gated ion channels (LGICs).

B. Membrane topology model for cystic fibrosis transmembrane regulator (CFTR). *R*, regulatory domain; NBD, nucleotide-binding domain.

(Adapted from Jentsch, 1996)

Hydropathy analysis predicts that CFTR contains structural domains similar to those in other proteins of the ATP-binding cassette (ABC) family. The membrane topology of CFTR (Figure 1-1B) includes twelve-predicted transmembrane helices (TM1 to TM12) in two groups of six, two cytoplasmic nucleotide-binding domains (NBDs), which are the sites of ATP hydrolysis, as well as a regulatory domain (R), which contains numerous consensus sequences for phosphorylation by PKC and PKA (Chang *et al.,* 1994; Jia *et al.,* 1997). In addition, a PDZ binding domain (a common motif for protein interactions), was also identified at the carboxyl terminal tail of CFTR (Wang *et al.,* 1998). The first protein reported to bind to the *C* terminus of CFTR was the ezrin binding phosphoprotein 50 (EBP50), which may act as an A kinase anchoring protein (Short *et al.,* 1998).

The question of whether CFTR functions as a monomer forming individual Cl^- channels, or whether the Cl^- conductance is a result of two or more proteins, is not clear yet. The recent, molecular cloning of the CFTR associated protein, CAP70, a PDZ domain protein, supports the CFTR dimer hypothesis (Wang *et al.,* 2000). Electrophysiological analysis of CFTR dimerisation by CAP70 showed that although there was no effect on the single channel conductance, the open probability of the CFTR channel increased.

In addition to the Cl^- channel activity, CFTR regulates various other channels through ATP release (reviewed by Hryciw and Guggino, 2000). These channels include the outwardly rectifying Cl^- channel (ORCC), the amiloride-sensitive Na^+ channel (ENaC) and the inwardly rectifying renal outer medullary K^+ channel, (ROMK).

1.2.3 CHLORIDE CHANNEL (ClC) FAMILY

During the last decade a new class of Cl^- channel proteins, the ClC family, has been identified through molecular cloning techniques. The ClC proteins form the largest gene family of Cl^- channels, and they show a high degree of evolutionary conservation. Mutational analysis of ClCs showed that defects of ClC sequence are responsible for a number of diseases (summarized in Table 1-1).

Using the electroplax organ of the marine ray *Torpedo,* which is highly enriched in voltage-sensitive Cl^- channels, Jentsch *et al.*, (1990) isolated a fraction of messenger RNA that was associated with an increase in Cl^- current when expressed in *Xenopus* oocytes. This study led to the cloning of the first member of the ClC family, ClC-0. Homology cloning has since resulted in the identification of 9 homologues within mammals. Mammals express three ClC subfamilies as shown in Figure 1-2. The first subfamily, which is most closely related to the *Torpedo* channel tClC-0 (Jentsch *et al.*, 1990), includes the skeletal muscle hClC-1 (Steinmeyer *et al.*, 1994; Meyer-Kleine *et al.*, 1995), the ubiquitously expressed rClC-2 (Grunder *et al.*, 1992), and the two kidney specific channels rClC-K1 and rClC-K2 (Kieferle *et al.*, 1994; Uchida *et al.*, 1995). The second subfamily, which is closely related to the putative *S. cerevisiae* yeast ClC channel (Huang *et al.*, 1994), includes the widely expressed channels rClC-3 and rClC-4 (Kawasaki *et al.*, 1994; Adachi *et al.*, 1994), and the kidney rClC-5 (Lloyd *et al.*, 1996; Steinmeyer *et al.*, 1995). The third subfamily includes the putative channel proteins hClC-6 and rClC-7 (Brandt and Jentsch, 1995). The ClC proteins encoded by each gene subfamily are closely related, whereas sequence identity between subfamilies is low.

ClC Subtype	Functional role	Human (murine) phenotype	References
ClC-1	Sarcolemma excitability	Myotonia congenita	Koch *et al.*, 1992
ClC-2	Epithelial Cl^- transport Cell volume regulation	Retina degeneration male infertility	Bosi *et al.*, 2001
ClC-3	Vesicles acidification Cell volume regulation	Neurodegeneration retina degeneration	Strobrawa *et al.*, 2001
ClC-4	Unknown		
ClC-5	Endosomal acidification	Dent's disease nephrolithiasis syndromes	Lloyd *et al.*, 1996
ClC-6	Unknown		
ClC-7	Bone resorbtion by osteoclasts	Infantile malignant osteporosis	Kornak *et al.*, 2001
ClC-Ka	Renal Cl^- reabsorption	Nephrogenic diabetes insibidus	Matsumura *et al.*, 1999
ClC-Kb	Renal Cl^- reabsorpion	Bartter's syndrome	Simon *et al.*, 1997

Table 1-1. Functional roles of mammalian ClC channels and related diseases.

Adapted from George *et al.*, (2001)

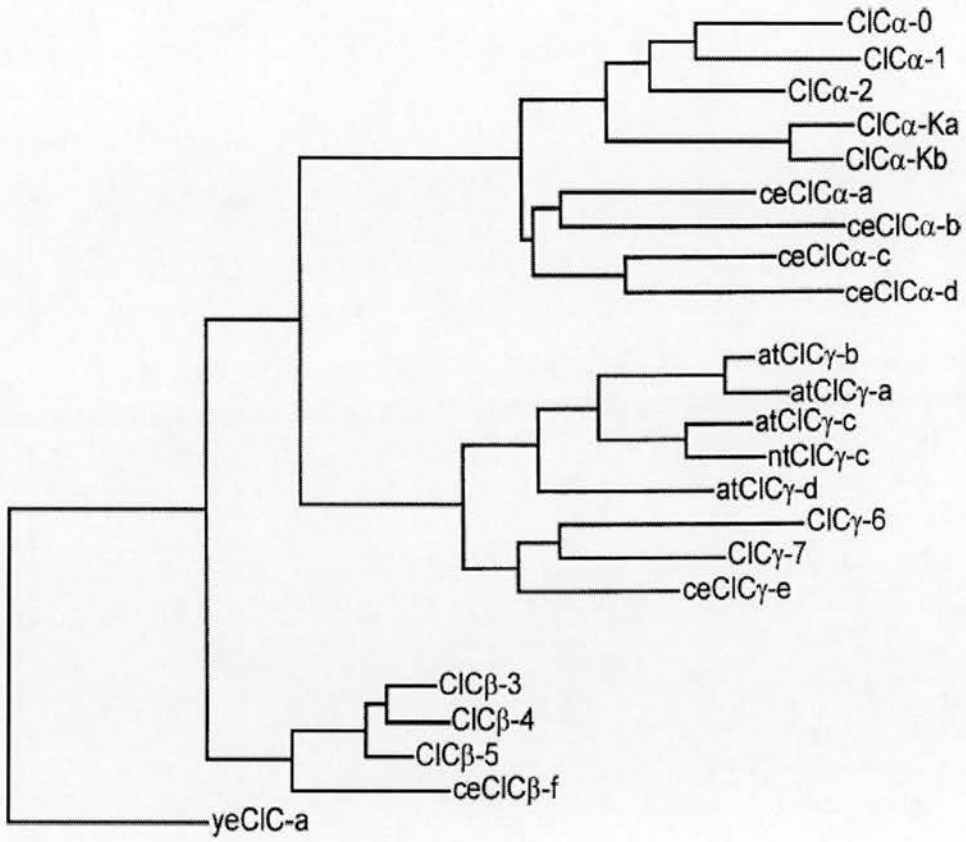


Figure 1-2. Chloride Channels (ClC) subfamily organisation.

Homology cloning has resulted in the identification of at least nine homologues within mammals, as well as homologues within bacteria and prokaryotes.

Prefixes: "ce" (*C. elegans*), "ye" (*S. cerivisiae*), "at" (*A. thaliana*).

(Adapted from Maduke *et al.*, 2000)

Genome sequencing evidence suggests that multiple ClC channels also exist in a wide range of other species from bacteria to *Drosophila*. The ClC genes in prokaryotes are known only from genome sequencing studies, and with the exception of the *Escherichia coli* ClC (Maduke *et al.*, 1999), nothing is known about these members beyond their predicted sequences.

The ClC proteins are α-helical membrane proteins (Purdy and Wiener, 2000), with 10-12 predicted transmembrane domains (Schmidt-Rose and Jentsch, 1997). Both the *N* and *C* terminal tails are cytoplasmic. Transmembrane helices 9-12 contain a particularly hydrophobic stretch of sequence that was proposed to cross the membrane three or five times but not four (Purdy and Wiener, 2000). The proposed topology of ClC proteins is shown in Figure 1-3.

All the members of the ClC family are gated by transmembrane voltage. ClC gating occurs also in response of other factors such as pH (ClC-0, ClC-1, ClC-4 and ClC-5), cell swelling (ClC-2 and ClC-3) or phosphorylation (ClC-3). The magnitude and the direction of the effect vary strongly between homologues and even within members of the same subfamily. The molecular mechanisms underlying ClC gating are unknown, and are presumably related to the structure of the ClC gating pore. In contrast to all other known α-helical ion channel proteins, which form single-pore oligomers of four-, five- or six-fold symmetry, ClC channel proteins are two-pore homodimers (Middleton *et al.*, 1996; Ludewig *et al.*, 1996). Although other models have been proposed (Fahlke *et al.*, 1998) recent evidence from a 6.5 Å resolution projection image from 2D crystals of a bacterial ClC support the two-pore homodimer model (Mindell *et al.*, 2001). Apart from the asymmetric nature of the

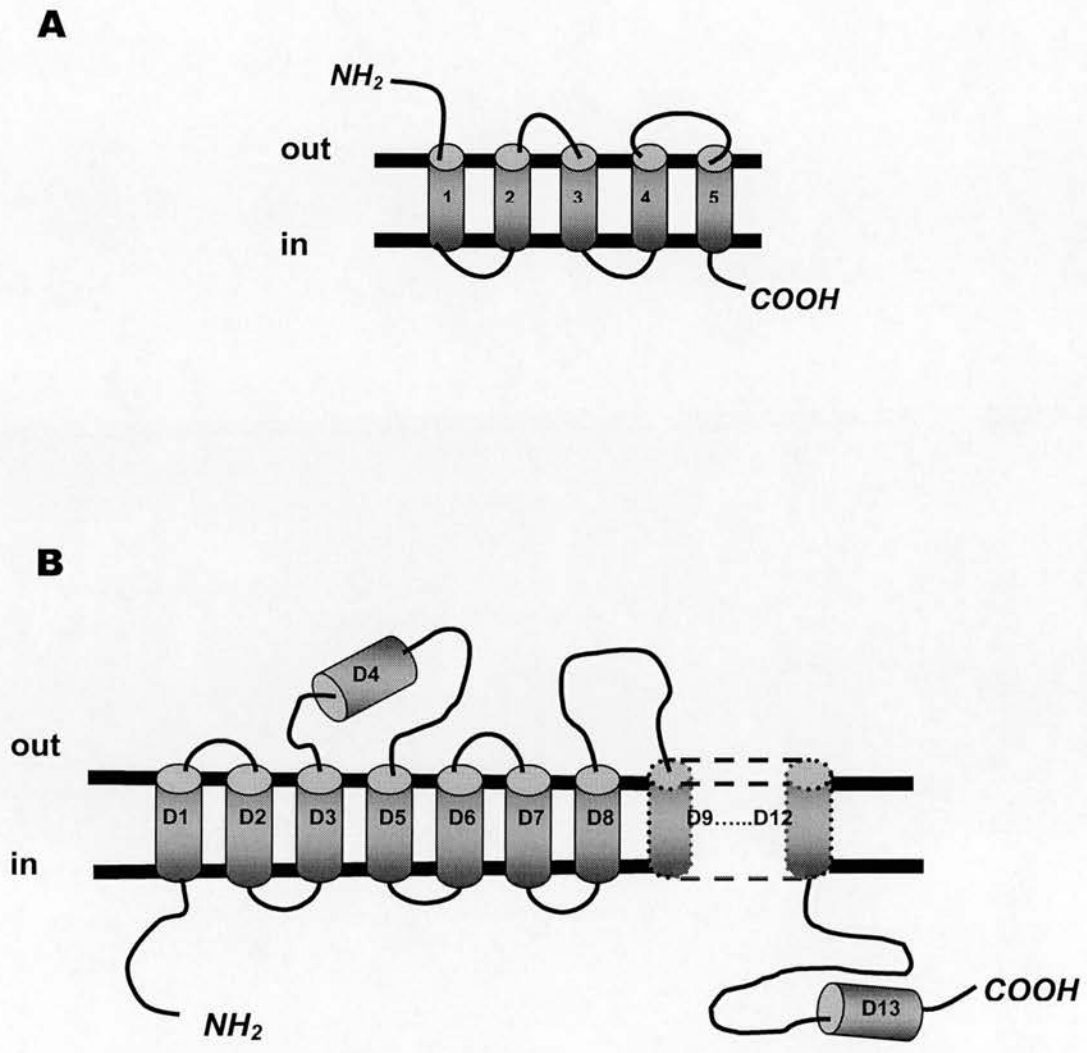


Figure 1-3. Proposed topologies of CaCC and ClC channels.

A. Membrane topology model of Ca^{2+} activated chloride channels (CLCA).

B. Membrane topology chloride channels (CLC, adapted from Jentsch, 1996).

ClC channel pore, its molecular basis is still unknown. Assuming that the pore-forming residues are conserved among members of the ClC family, Fahlke *et al.*, (1997) proposed that these residues ('hotspots') form part of a selectivity filter lining the channel pore.

ClC-0 is the only member of the ClC family proved to function alone as a Cl^- channel. Until recently, no associated partners for ClCs have been identified which could facilitate understanding of the molecular basis of ClC-gating. However, Estevez and his co-workers (Estevez *et al.*, 2001) identified the transmembrane protein, barttin, as a β subunit for ClC-K Cl^- channels, crucial for their function. Barttin was originally identified after positional cloning of the Bartter's syndrome type 4-associated gene (BSND; Birkenhager *et al.*, 2001). Electrophysiological studies in combination with mutation experiments revealed that when barttin was co-expressed with ClC-K homologues (ClC-Ka and ClC-Kb) in *Xenopus* oocytes larger currents were generated. Furthermore, *in situ* hybridisation and immunofluorescence analysis in nephron confirmed co localisation of barttin and ClC-K proteins.

1.2.4 Ca^{2+} ACTIVATED CHLORIDE CHANNEL (CLCA) FAMILY

The calcium activated chloride channel (CLCA) family is the most recently identified family of plasma membrane Cl^- conductance proteins (reviewed by Gruber *et al.*, 2000; Fuller and Benos, 2000; Pauli *et al.*, 2000). To date ten members of the CLCA family have been described. These include the two bovine homologues bCLCA1 (bCaCC; Ran *et al.*, 1992; Cunningham *et al.*, 1995) and bCLCA2 (Lu-ECAM 1 – lung endothelial cell adhesion molecule 1; Elble *et al.*, 1997); three

murine homologues mCLCA1 (Gandhi *et al.*, 1998), mCLCA2 (Lee *et al.,* 1999), mCLCA3 (gob-5; Komiya *et al.*, 1999); and four human homologues, hCLCA1 (Gruber *et al.*, 1998a), hCLCA2 (Gruber *et al.*, 1999), hCLCA3 (Gruber *et al.*, 1999), and hCLCA4 (Agnel *et al.*, 1999). In addition, sequence fragments from two porcine homologues and one from rat have been included in the Genebank database.

The first member of the CLCA family, bovine CLCA1 (bCLCA1), was affinity purified from bovine tracheal apical membrane vesicles and biochemically characterised as a 140 kDa complex (Ran and Benos, 1992). Reconstitution of the purified protein into planar lipid bilayers revealed that the protein behaved as an anion channel (Ran *et al.*, 1992) that is regulated by Ca^{2+}, via a calmodulin kinase II-dependent mechanism (Fuller *et al.*, 1994). Following the molecular cloning of bCLCA1, an outward rectifying 4,4'-diisothiocyanostilbene-2, 2'-disulfonic acid (DIDS)-sensitive anion channel was detected in *Xenopus* oocytes, when they were injected with bCLCA cRNA (Cunningham *et al.*, 1995). In the same study, a Ca^{2+}-dependent anion conductance was observed in COS-7 cells transfected to produce the recombinant protein. Patch-clamp studies have shown that three other homologues of the CLCA family (hCLCA1, hCLCA2 and mCLCA1) exhibit similar characteristics to those described for bCLCA1 such as activation by Ca^{2+}, inhibition by DIDS and DTT (Gandhi *et al.*, 1998; Gruber *et al.*, 1998b; Gruber *et al.*, 1999).

Unlike some ClC members and the CFTR channel, members of the CLCA family appear to be expressed with a high degree of tissue specificity. The two ``founder members``, bCLCA1 and bCLCA2, are expressed in respiratory epithelia of the trachea and bronchi and in the epithelia of the aorta and pulmonary. Human

homologues hCLCA1 and hCLCA4 and murine homologues mCLCA1 and mCLCA3 are expressed in intestinal epithelia, hCLCA3 and mCLCA1 are expressed in respiratory epithelia; hCLCA1, hCLCA4 and mCLCA1 are expressed in uterus, prostate, epididymis and testes, hCLCA1, mCLCA1 and mCLCA2 are expressed in mammary epithelium, and hCLCA4 is expressed in brain.

The protein expression, processing and primary structure of CLCA proteins have been studied in detail in bCLCA2 (Elble *et al.*, 1997). These protein characteristics were shown to be generally conserved among the other CLCA proteins. With the exception of hCLCA3, all the known CLCA members encode an approximately 125 kDa precursor transmembrane protein with one proteolytic cleavage site distinct from the amino terminal signal sequence. Cleavage of the precursor produces a 90 kDa protein segment with four putative transmembrane domains and a 30-40 kDa protein segment. Other conserved features include the highly symmetrical cysteine-rich motif, $CX_{12}CX_4CX_{12}C$, in the amino terminal tail; five to eight *N*-linked glycosylation sites; consensus phosphorylation sites for PKC, and two highly hydrophobic regions at the *N* and *C* termini (Figure 1-3 A). An additional feature of this family is that the CLCA members may be multifunctional proteins, similar to CFTR. Two CLCA proteins, bCLCA2 and mCLCA1, were originally described as cell-cell adhesion molecules mediating lung colonisation of lung metastatic murine B16-F10 melanoma cells (Zhu *et al.*, 1991). Furthermore, hCLCA2 (Szabo *et al.*, 1998) and mCLCA2 (Elble and Pauli, 2001) have been implicated in tumor supression in breast cancer cells. The mechanism by which hCLCA2 and mCLCA2 carry out this role still remains unknown.

1.3 ION CHANNELS OF INTRACELLULAR ORGANELLES

Members of the Cl^- channel families discussed so far are mainly present in plasma membrane. Due to advances in electrophysiological techniques including patch clamping, reconstitution into lipid bilayers, and other macroscopic flux measurements, ion channels from intracellular organelle membranes (endoplasmic / sarcoplasmic reticulum, mitochondria, nucleus, synaptic vesicles, chromaffin and zymogen granules) have also been identified and analysed. In this section, intracellular calcium and potassium channels are briefly discussed, followed by a more detailed analysis of intracellular Cl^- channels with particular reference to the most recent Cl^- channel family of intracellular organelles, CLIC (Edwards, 1999).

1.3.1 INTRACELLULAR CALCIUM CHANNELS

Two major families of intracellular channel proteins are responsible for releasing Ca^{2+} from intracellular stores: inositol 1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs; reviewed by Berridge, 1993; Clapham, 1995; McPherson and Campbell, 1993; Mikoshiba, 1993). Multiple isoforms and subtypes (generated by alternative mRNA splicing) of each of the above Ca^{2+}-release channel (CRC) class display distinct but overlapping distributions within mammalian tissues. RyRs were first identified in muscle cells because of their high affinity for the plant alkaloid, ryanodine. They are now known to function in Ca^{2+} signalling in a wide variety of muscle and non-muscle cell types (Meissner, 1994; McPherson and Campbell, 1993; Coronado *et al.*, 1994). IP_3Rs are primarily expressed in brain but they are also found in locations such as the smooth muscle of arteries, the oviduct

and uterus. They are largely located on the surface of the ER, but may also be distributed in other intracellular membranes *e.g.* the inner nuclear membrane. The primary structure of SCaMPER (sphingolipid Ca^{2+}-release-mediating protein from endoplasmic reticulum), a third candidate class of CRC, has also been reported (Mao *et al.*, 1996), although the properties of this channel have not been thoroughly investigated.

RyRs and IP_3Rs display approximately 70 % amino acid identity between isoforms within each family, with limited identity between the two families (MacKrill, 1999). IP_3Rs contain three domains: an IP_3-binding *N* terminal domain, a regulatory domain containing ATP binding and phosphorylation sites, and a *C* terminal domain containing six transmembrane regions (Taylor and Richardson, 1991). Binding of IP_3, as well as Ca^{2+}, to the *N* terminal domain induces a conformational change in the protein leading to Ca^{2+} channel opening. The transmembrane regions are responsible for the aggregation of four subunits into the functional tetrameric receptor protein, and also serve to form the Ca^{2+} channel. Electron microscopy and image reconstruction of both CRC proteins support the concept of a tetrameric protein of pinwheel-like structures with four radial arms protruding from a central hub (Chadwick *et al.*, 1990).

1.3.2 INTRACELLULAR POTASSIUM CHANNELS

Electrophysiological studies revealed the presence of potassium (K^+) channels in various intracellular organelles. Sarcoplasmic reticulum (SR) membranes in skeletal and cardiac muscle contain K^+-selective channels, which are thought to help charge compensation during Ca^{2+} transport by providing a pathway for counter-charge movement during muscle contraction and relaxation (Miller and Racker, 1976; Shen *et al.*, 1990; Sokabe *et al.*, 1991). The K^+-selective channel in cardiac muscle SR (Mr ~80 kDa) has a large conductance (150 pS), with sub-conducting states, and its open probability increases with depolarising voltages (Rousseau *et al.*, 1992). The activity of this channel is not modified by ATP, GTP, cAMP, or by the phosphorylation conditions (Rousseau *et al.,* 1992), but it seems to be regulated via various types of Ca^{2+} binding sites present on the channel molecule (Uehara *et al.*, 1994). The K^+ channel observed in skeletal muscle SR has also a large conductance (Hirashima *et al.*, 1991). The activity of this channel is not affected by physiological Ca^{2+} concentrations, or by Mg^{2+} (Wang and Best, 1994; Picher *et al.*, 1996).

A K^+ selective ATP-sensitive ion channel (mito K_{ATP} channel) has been discovered in the inner membrane of rat liver mitochondria and reconstituted into proteoliposomes (Paucek *et al.*, 1992). Briefly, ATP inhibits the mito K_{ATP} channel (Ashcroft and Ashcroft, 1992), whereas GTP and GDP reverse the ATP-inhibition of the mito K_{ATP} channel (Paucek *et al.*, 1996).

The presence of large conductance (150-300 pS) Ca^{2+} dependent K^+ selective channels (K^+_{CG} channel) has been identified in the membranes of chromaffin granules after fusion of granule vesicle with planar bilayer membrane (Arispe *et al.*,

1992; Ashley *et al.*, 1994). K^+ channel activity has also been observed in the membranes of isolated pancreatic zymogen granules (Thevenod *et al.*, 1992; Gasser and Holda, 1993). Similar to the plasma membrane K_{ATP} channels, the K^+ conductance of pancreatic zymogen granule membrane is inhibited by ATP and antidiabetic sulfonyureas (like glibenclamide), and activated by K^+ channel openers (Thevenod *et al.*, 1994; Thevenod *et al.*, 1996).

K^+ channels are also present in the nuclear envelope (Mazzanti *et al.*, 1990; Matzke *et al.*, 1990; Bustamante, 1992; Mazzanti *et al.*, 1994; Rousseau *et al.*, 1996; Assandri and Mazzanti, 1997). Interestingly, a voltage- and Ca^{2+}-activated K^+ channel has been reported in the outer nuclear envelope isolated from pancreatic acinar cells (Maruyama *et al.*, 1995). These channels, which contribute to the nuclear membrane potential, may be important in balancing the charge carried by the movement of macromolecules in and out of the nucleus.

1.3.3 INTRACELLULAR CHLORIDE CHANNELS

Cl^- channels are probably found in every cell, from bacteria to mammals. Due to technical difficulties, the study of Cl^- channels has lagged behind that of the cation selective ion channels. With the advent of the patch clamp technique, many of these technical problems have been overcome and the Cl^- channel field has been advancing steadily.

Cl^- channels are found in a variety of intracellular organelles such as Golgi, endosomes, endoplasmic/sarcoplasmic reticulum, mitochondrial and nuclear membrane, synaptic vesicles and chromaffin and zymogen granules. Later in this report, a brief description of the different types of Cl^- channels is given for each of these intracellular organelles.

1.3.3.1 CHLORIDE CHANNELS OF SR AND ER MEMBRANES

Two SR anion channels have been identified in rabbit skeletal muscle. The high conductance BCl channel (250 pS in 250/50 mM Cl^-; *cis/trans*) is apparently unregulated by cytoplasmic ligands and is constitutively open in lipid bilayers (Kourie *et al.*, 1996). The smaller conductance SCl channel (75 pS in 250/50 mM Cl^-) is highly regulated, and partially inhibited by cytoplasmic acidification (< pH7), millimolar concentrations of adenine nucleotides (Ahern and Laver, 1998; Kourie, 1999) and inositol phosphates (Kourie *et al.*, 1997). It is voltage dependent (Kourie *et al.*, 1996), activated and inhibited by oxidation and reduction, respectively (Kourie, 1997) and activated by ~1 μM cytoplasmic Ca^{2+} (Kourie *et al.*, 1996). In muscle there appears to be an excess of SR Cl^- channels even though Cl^- is not an important counter ion during Ca^{2+} release from SR (Coonan and Lamb, 1998). The role of anion channels in the SR is unknown. It had been suggested that their role might be to contribute a Cl^- flux to the counter current during Ca^{2+} release and uptake (Kourie *et al.*, 1996). However, the failure to demonstrate changes in SR [Cl^-] during muscle tetanus (Somlyo *et al.*, 1981) and the lack of an effect of Cl^- removal on voltage sensor-activated contraction (Coonan and Lamb, 1998) suggest that Cl^- is not

an essential contributor to counter currents. It is more likely that the role of the anion channels is associated with the transport of other relatively permeant anions (*e.g.* SO_4^{-2} and P_i).

Cl^- channels are also present in the SR of cardiac muscle. The main characteristics of these channels are their activation by protein kinase A (PKA)-dependent phosphorylation, inhibition by Ca^{2+}-calmodulin and regulation by cytoplasmic pH (Townsend and Rosenberg, 1995; Kawano *et al.*, 1999). Kawano *et al.,* (1999) suggested that the SR Cl^- channel in cardiac cells might serve as a transporter for the movement of adenine nucleotides between the cytosol and the SR lumen.

Cl^- channel activity has also been observed in ER membranes from rat exocrine pancreas (Schmid *et al.*, 1988), and from rat and sheep brain (Ashley, 1989b; Silvestro and Ashley, 1994; Howell *et al.*, 1996; Clark *et al.*, 1997). A Poisson analysis strongly supported the hypothesis that the ryanodine-sensitive Ca^{2+} channels discovered in rat brain ER (Ashley, 1989a) are co-localised with Cl^- channels. These channels appear to be poorly selective, consistent with their role in charge compensation. Recently, confocal imaging and dual experiments of COS and CHO cells co-transfected with Cl^- channel proteins ClC-6a or -6c and the sarco/endoplasmic reticulum Ca^{2+} pump (SERCA2b) indicated that the ClC-6 isoforms co-localise with the Ca^{2+} pump (Buyse *et al.*, 1998). Thus it was suggested that ClC-6a and 6c act as intracellular membrane Cl^- channels proteins, most likely residing in the ER.

1.3.3.2 CHLORIDE CHANNELS OF THE MITOCHONDRIAL MEMBRANE

Mitochondria inner and outer membranes contain various ion channels (Sorgato and Moran, 1993). The activity of intracellular channels from mitochondria was first observed directly using the patch clamp technique (Sorgato *et al.*, 1987). The most structurally and electrophysiologically characterised mitochondrial channel is the voltage-dependent anion channel (VDAC; reviewed by Mannella *et al.*, 1992; Colombini *et al.*, 1996). It seems that mitochondrial membrane contain anion selective channels rather than strictly Cl^- selective channels. Reconstitution of sheep cardiac mitoplasts (inner mitochondrial membrane vesicles) into planar lipid bilayers revealed two distinct activities, one of intermediate conductance and one of low conductance. Both of these states discriminate poorly between anions and cations. (Pollard *et al.*, 1979; Hayman and Ashley, 1993; Hayman *et al.*, 1993)

1.3.3.3 OTHER INTRACELLULAR CHLORIDE CHANNELS

Anion channels are also present in other intracellular organelles. The presence of Cl^- channels has been demonstrated in the nuclear envelope (Mazzanti *et al.*, 1990; Matzke *et al.*, 1990; Bustamante, 1992; Mazzanti *et al.*, 1994; Valenzuela *et al.*, 1997). Similar to K^+ channels, nuclear Cl^- channels contribute to the nuclear membrane potential, and possibly facilitate the charge compensation as macromolecules move into and out of the nucleus. Cl^- channel activity has also been detected in Golgi and endosomes (Glickman *et al.,* 1981), as well as in bovine

chromaffin granule membranes (Pollard *et al.*, 1979), probably associated with the large dense core vesicles (LDCVs; Chuang *et al.*, 1999).

1.4 CHANNEL PROTEIN OR CHANNEL REGULATOR

A number of criteria need to be met if an identified protein is to be considered a channel protein. Among all other criteria, homology with a previously identified channel protein is useful but not always possible. A channel protein should contain transmembrane domains, with two transmembrane domains being the smallest number generally associated with a proven channel protein. Expression of the putative channel protein in heterologous systems combined with the patch-clamping technique is very useful in channel identification, providing that a similar conductance is not already expressed in the experimental system. Further electrophysiological studies such as incorporation of purified protein into planar lipid bilayers and single channel recordings, together with the appropriate controls are also useful. Finally mutations that affect the ion selectivity or conductance of the putative channel protein can further provide evidence for the correct characterisation of the channel activity of a protein.

All the channel proteins discussed thus far have satisfied the above criteria. However, there are at least four other proteins and their homologues that have been proposed to associate with Cl^- channel activity in intracellular and/or plasma membranes when they are expressed in heterologous systems. These are phospholemman, MAT-8, sucrase-isomaltase (SI) homologue and some members of the p64/CLIC superfamily.

Phospholemman (PLM) is a small (15 kDa) membrane protein originally isolated from canine cardiac sarcolemmal membranes (Palmer *et al.*, 1991). Phospholemman is a widely expressed protein with a single transmembrane domain, a large cytoplasmic *C* terminal and an extracellular *N* terminal domain. PLM contains consensus phosphorylation sites for both cAMP-dependent protein kinase A and protein kinase C in its cytoplasmic domain, and seems to act as a channel when expressed in *Xenopus* oocytes (Moorman *et al.*, 1992; Mounsey *et al.*, 1999). Although it has been described as a Cl^- channel, over-expression of the protein has also been associated with cationic and anionic currents. It has been recently suggested that phospholemman may serve a role in cell volume regulation as it was found that it can conduct taurine and possible other osmolytes (Morales-Mulia *et al.*, 2000).

A novel PLM-like protein, MAT-8 has been isolated from human mammary carcinoma (Morrison *et al.*, 1995). MAT-8 shares some sequence homology and membrane topology with PLM, but has a different expression pattern as determined by Northern hybridisation analysis. In contrast to PLM, the cytoplasmic domain of Mat-8 contains no consensus phosphorylation sites. Expression of Mat-8 in *Xenopus* oocytes induces hyperpolarisation-activated Cl^- currents similar to those induced by PLM expression. Both PLM and MAT-8 may be pore forming subunits of a Cl^- channel protein, or may play a modulatory role upon association with functional membrane partners.

The latest addition to the growing family of the identified and proposed Cl^- channels is a homologue of the intestinal digestive enzyme sucrase-isomaltase (SI; Hunziker *et al.*, 1986). SI-like Cl^- channel was affinity purified using a monoclonal antibody against a protein isolated from the *Necturus* (mudpuppy) gallbladder and found to be a 260 kDa membrane protein that contains a single transmembrane domain (Finn *et al.*, 2001). When the SI-like protein was heterologously expressed in CHO cells, a cAMP-dependent Cl^- sensitive current was observed using whole-cell patch clamping. Similar currents were observed when the protein was expressed in the *Xenopus* oocytes system, and these currents were blocked by the monoclonal antibody used to purify the protein.

Probably the most well characterised example of a regulator membrane protein is that of phospholamban (lamban - from the Greek word $\lambda\alpha\mu\beta\acute{\alpha}\nu\omega$ = *accept* phosphate group). Phospholamban (PLB) is a small 52 amino acid integral membrane phosphoprotein that regulates the sarcoplasmic reticulum Ca^{2+}-transport ATPase (SERCA2α isoform) in cardiac muscle (Tada *et al.*, 1975). The phospholamban self-assembles into pentamers in SDS solution and in lipid bilayers (Wegener and Jones, 1984; Cornea *et al.*, 1997). Phospholamban monomer is organised into three physical and functional domains: two helical domains IA and II connected by a less-structured domain IB (Terzi *et al.*, 1992; Simmerman *et al.*, 1989). The mechanism by which SERCA2α is controlled by phospholamban relies on the phosphorylation states of phospholamban (reviewed by Colyer, 1998). In the dephosphorylated state, phospholamban inhibits the Ca^{2+}-ATPase by decreasing its apparent Ca^{2+} affinity. Phosphorylation of phospholamban by cAMP- or Ca^{2+}-calmodulin-dependent protein kinases respectively, restores the high affinity of

ATPase for Ca^{2+}, and thus increases Ca^{2+} transport into cardiac sarcoplasmic reticulum. Similar results were observed upon binding of anti-PLB monoclonal antibodies to the cytoplasmic phosphorylation domain (I_A) of phospholamban This inhibitory effect of PLB on the Ca^{2+}-ATPase, has been suggested to involve the reversible binding of PLB to the Ca^{2+}-ATPase (James *et al.*, 1989).

1.5 CHLORIDE INTRACELLULAR CHANNEL (CLIC) FAMILY

Recently, several related genes that share remarkable homology with the *C* terminal half of bovine p64 (Landry *et al.*, 1993) have been cloned (Figure 1-4). These proteins constitute a protein family called CLIC (chloride intracellular channel) proteins. Members of CLIC family include CLIC1 (Valenzuela *et al.*, 1997), CLIC2 (Rogner *et al.,* 1996), CLIC3 (Qian *et al.,* 1999), CLIC4 (Duncan *et al.*, 1997), CLIC5 (Berryman and Bretscher, 2000) and the p64-related protein, Parchorin (Nishizawa *et al.*, 2000). In addition, the osteoclast ruffled border Cl^- channel (p62) has been reported to be antigenically related to p64, the founder member of CLIC family (Schlesinger *et al.*, 1997). Although each of the CLIC family members shows high homology to the *C* terminal half of p64, none of the CLICs have a region with significant homology to the *N* terminus of p64, suggesting that the putative channel activity must reside in a region within the *C* terminus. Sequence analysis of CLIC members has revealed a number of phosphorylation motifs for protein kinase C, cAMP dependent protein kinase, casein kinase II and tyrosine kinase (mainly residing within the *C* terminal domain) as well as sequence similarity with Omega class GSTs (Dulhunty *et al.*, 2000).

CLIC proteins exist in both soluble and membrane-bound form. Although this dual localisation is unusual for channel-forming proteins, it has been previously observed with other intracellular ion channel proteins such as annexins (Doubell *et al.*, 1993; Clemen *et al.*, 1999) and Bcl-x(L) (Minn *et al.*, 1997). CLIC proteins share a wide tissue and subcellular distribution, suggesting that members of this gene family may have related but distinct functions.

Various electrophysiological studies have shown that several members of this gene family, including bovine p64, CLIC1, CLIC3 and rat brain CLIC4 play a role in Cl^- transport, possibly directly or indirectly. With the exception of CLIC2, all members of the CLIC family have been characterised at the molecular level and to some extent at the biochemical level, as described below.

1.5.1 p64

p64 was originally identified after partial purification of a 64 kDa protein from bovine kidney microsomes by ligand affinity chromatography using an indanyloxyacetic acid (IAA) alternative as a high affinity synthetic ligand (Landry *et al.*, 1989). Bovine p64 was cloned and found to be a 428-amino acid protein consisting of a hydrophilic *N* terminal domain and a *C* terminal domain containing two predicted transmembrane domains (Landry *et al.*, 1993).

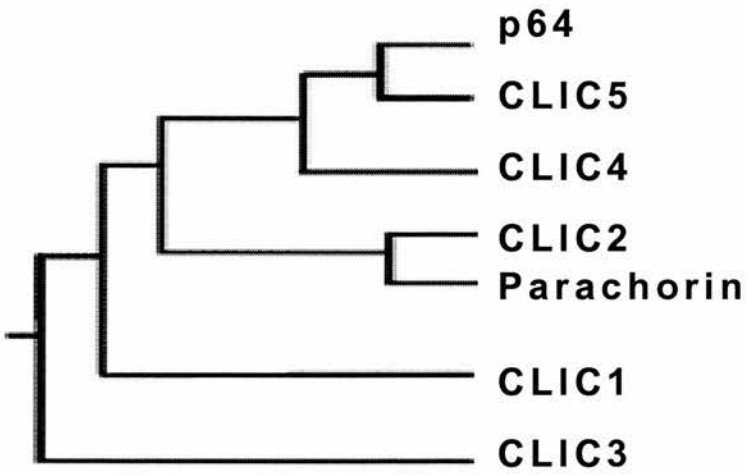


Figure 1-4. Phylogenetic tree of the CLIC and p64-related proteins.

Sequence alignment of the CLIC proteins and the C terminal half of p64-related proteins, was performed using the Clustal method. (MegAlign, DNASTAR software).

Following the molecular cloning and characterisation of p64 (Landry *et al.*, 1993), further insights into the subcellular distribution and targeting of p64 were reported by Redhead *et al.*, (1997). In this study p64 was localised by indirect immunofluorescence into intracellular vesicles distinct from the fluid phase or receptor-mediated endocytic compartments as shown by double staining with endocytic markers, transferrin receptor and endocytosed fluorescent dextran. Upon treatment of T84 cells (a human colon cancer cell line), which express endogenous p64 and p64-transfected PancI cells (a human cancer cell line) with phorbol 12-myristate 13-acetate (PMA), the protein kinase C activator, and with A23187, the Ca^{2+} ionophore, Redhead *et al.*, showed that p64 immunofluorescence staining moved from the cell centre towards the cell periphery. However the distribution of p64 fluorescence was unaffected when cells were treated with forskolin, the cAMP protein kinase activator. The conclusion from these data was that p64 is located in a secondary messenger-dependent secretory pathway, which is regulated by both Ca^{2+} and PKC but not by cyclic AMP.

Targeting of p64 to this distinct class of secretory vesicles was investigated using mutagenesis experiments. To determine whether the *C* terminus is important in p64, targeting mutants and chimeric proteins were generated. In generating the chimeric proteins, the *C* terminal domain of a natural plasma membrane protein, CD4, was replaced with the *C* terminus of p64. The subcellular distribution of p64 was then followed by immunostaining with p64 affinity purified antibody. The conclusion from these experiments was that *C* terminal domain prevents expression of p64 in the plasma membrane. Similar mutagenesis experiments in the *N* terminal domain of p64 demonstrated that the *N* terminus is necessary to deliver p64 to the

appropriate membrane compartment. Thus both the *N* and *C* terminal domains of p64 contribute to the subcellular localisation of the protein (Redhead *et al.*, 1997). Furthermore, when exogenous p64 was expressed in *Xenopus* oocytes, it was excluded from the plasma membrane. This supports the localisation of p64 to intracellular membranes (Redhead *et al.*, 1992; Redhead *et al.*, 1997).

Electrophysiological studies suggested that a novel Cl^- channel activity was associated with the expression of p64 in HeLa cells. The p64 associated activity appeared to be strongly anion selective, rectifying, phosphatase-activated and inhibited by DNDS (4,4'-dinitrostilbene-2-2'-disulphonic acid) and calixarene (Edwards *et al.*, 1998). In the same study, preliminary evidence was reported on the orientation of p64 in membrane vesicles (using a combination of phosphorylation and bilayer experiments), suggesting outward rectification if the channels were exposed on the plasma membrane (Edwards *et al.*, 1998).

Functional characterisation of p64 by Edwards and Kapadia (2000) revealed that the channel activity of p64 is regulated by $p59^{fyn}$, a member of the Src family of tyrosine kinases. Co-expression of p64 with $p59^{fyn}$ in HeLa cells led to tyrosine phosphorylation of p64. To determine whether p64 is a ligand for Fyn, lysates from cells expressing p64 and $p59^{fyn}$, or p64 alone, were incubated with immobilised GST-Fyn. These experiments showed that p64 only becomes ligand for the SH2 domain of Fyn, when it is tyrosine phosphorylated by $p59^{fyn}$. Similar, when native p64 from solubilised bovine kidney membranes was incubated with immobilised GST-Fyn SH2 fusion protein, binding was still detected but at lower levels. Upon treatment of solubilised membranes from cells co-expressing p64 and $p59^{fyn}$ with

alkaline phosphatase, p64 loses its affinity for Fyn-SH2. Using site-directed mutagenesis, the authors showed that the phosphorylation of tyrosine 33 is responsible for p64-FynSH2 binding, while mutations of tyrosine 20 and 56 had no effect on SH2 binding.

Reconstitution experiments were performed to determine whether p64-FynSH2 binding had a functional significance on the previously described p64 Cl^- channel activity (Edwards *et al.*, 1998). Membranes prepared from HeLa cells expressing p64 either alone or in combination with $p59^{fyn}$ were solubilised and reconstituted, and the Cl^- channel activity was assayed using a Cl^- sensitive electrode. Co-expression of p64 with $p59^{fyn}$ resulted in enhanced channel activity compared to reconstituted membranes from cells expressing p64 only. In contrast, co-expression of p64 with a mutant $p59^{fyn}$ that had lost its tyrosine kinase activity but retained its SH2/SH3 binding capacity failed to enhance Cl^- channel activity. Furthermore dephosphorylation of p64 by alkaline phosphatase, or mutation of tyrosine 33 to alanine failed to enhance the Fyn-dependent channel activation of p64. The conclusion from those data was that phosphorylation of p64 has a positive effect on p64 channel activity by enhancing the Cl^- efflux rate. However, is not clear whether p64 phosphorylation alone is sufficient, or whether subsequent binding of the phosphorylated p64 to Fyn-SH2 is important for p64 activation (Edwards and Kapadia, 2000).

1.5.2 CLIC1 (NCC27)

CLIC1 cDNA was originally identified by screening a cDNA library for genes associated with the activation of monocytoid cells (Valenzuela *et al.*, 1997). CLIC1 cDNA (or NCC27 for nuclear chloride channel of 27 kDa as it was originally named) encodes a 241-amino acid transmembrane protein homologous to the *C* terminus of bovine p64 protein (Landry *et al.*, 1993), with the *N* terminus orientated outward (Tonini *et al.*, 2000). Northern blot analysis of CLIC1 mRNA revealed a wide tissue distribution, similar to other members of the CLIC family. CLIC1 mRNA is expressed in almost all foetal and adult human tissues especially in brain, lung, liver, skeletal muscle, and in thymus. Similar mRNA distribution was also observed in mouse embryonic tissues (Tulk and Edwards, 1998; Valenzuela *et al.*, 2000).

Further analysis of the CLIC1 protein sequence revealed a number of consensus phosphorylation motifs for PKA, PKC and casein kinase II, single *N*-glycosylation and *N*-myristoylation sites, and two putative nuclear localisation sequences (KRR and KKYR). Immunoblotting and immunofluorescence analysis of CLIC1-transfected CHO-K1 cells (Chinese hamster ovary cells) demonstrated that the protein is mainly localised in the nucleus probably in a free soluble form in the nucleoplasm or in association with the nuclear membrane (Valenzuela *et al.*, 1997).

The channel activity of the protein was tested using whole-cell and single-channel patch clamping of both plasma membrane and isolated nuclei of CLIC1 transfected CHO-K1 cells (Valenzuela *et al.*, 1997; Valenzuela *et al.*, 2000; Tonini *et al.*, 2000). These studies showed that transfection of CHO-K1 cells with CLIC1 resulted in a Cl^--selective ion channel activity, not normally present in non-

transfected cells, with a permeability sequence of $SCN^->F^->Cl^->I^-$. Furthermore, treatment of the cells with pharmacological Cl^- channel blockers revealed that CLIC1 conductance was completely and reversibly blocked by IAA-94 whereas DIDS had no effect (Valenzuela *et al.*, 2000). In addition to these observations, it was shown that native CLIC1 Cl^- conductance occurs only in G2/M phase of the cell cycle, suggesting a role for CLIC1 in cytokinesis and re-organisation of the nuclear membrane during mitosis, possibly by regulating swelling of the cell and nucleus (Valenzuela *et al.*, 2000).

As reported previously, a number of CLIC members show sequence similarity to members of the glutathione-S- transferases (GSTs), which catalyse the conjunction of glutathione to many exogenous and endogenous compounds (Hayes and Strange, 1995). Detailed alignment investigation of an Omega class member of GST superfamily, GSTO1-1 (Board *et al.*, 2000), and CLIC1 sequence and subsequent homology modelling revealed conservation of key residues and strongly supported a folding similarity between these two proteins (Dulhunty *et al.*, 2001). Although GSTO1-1 failed to form channels, it was shown that it functions as a modulator of ryanodine-sensitive Ca^{2+} channels suggesting a possible role for GSTO1-1 in regulating intracellular Ca^{2+} (Dulhunty *et al.*, 2001). Recently, the crystal structure of the soluble form of CLIC1 has been resolved at 1.4 Angstroms resolution (Figure 1-5; Harrop *et al.*, 2001) providing evidence for the structural and folding similarity between GSTO1-1 and CLIC1. Further analysis of the crystal structure revealed that CLIC1 has an intact glutathione (GSH) binding site, conserved among other members of the CLIC family. This site has a redox-active cysteine, similar to that of glutaredoxin, and is capable of forming a covalent mixed

disulfide with glutathione, suggesting that CLIC channel activity may be under the control of redox-active signalling molecules.

At least one other binding partner has been identified for CLIC1 so far. Using the yeast-two-hybrid system and immunofluorescence it was shown that CLIC1 interacts and co-localises with AKAP350A a splice variant of the A Kinase Anchoring Protein 350 (AKAP350; Schmidt *et al.*, 1999) that bind the regulatory subunit of protein kinase A type II (Schmidt *et al.*, 2000). AKAPs are scaffolding proteins that maintain multivalent signalling complexes by binding enzymes including kinases and phosphatases (reviewed by Edwards and Scott, 2000).

1.5.3 CLIC2 (XAP121)

CLIC2 (XAP121) was identified during transcriptional analysis of the incontintinentia pigmenti (IP2) region Xq28 chromosomal band as a potential candidate gene for a number of diseases linked to this region (Rogner *et al.*, 1996). The isolated CLIC2 cDNA comprises of 1229 bp and its open reading frame (222-953) encodes a protein of 243 amino acids. Although no signal was detected by Northern blotting, reverse transcriptase PCR (RT-PCR) analysis revealed low expression levels in foetal liver and adult skeletal muscle (Rogner *et al.*, 1996). BLASTIN analyses of CLIC2 cDNA showed sequence homology with a number of human expression tags (ESTs), which in turn overlapped with bovine p64 and human CLIC1 (NCC27) Cl^- channel cDNAs. The attempt to clone the full length CLIC2 cDNA by Heiss and Poustka (1997) was unsuccessful. The molecular and biochemical characterisation of CLIC2 remains still to be investigated.

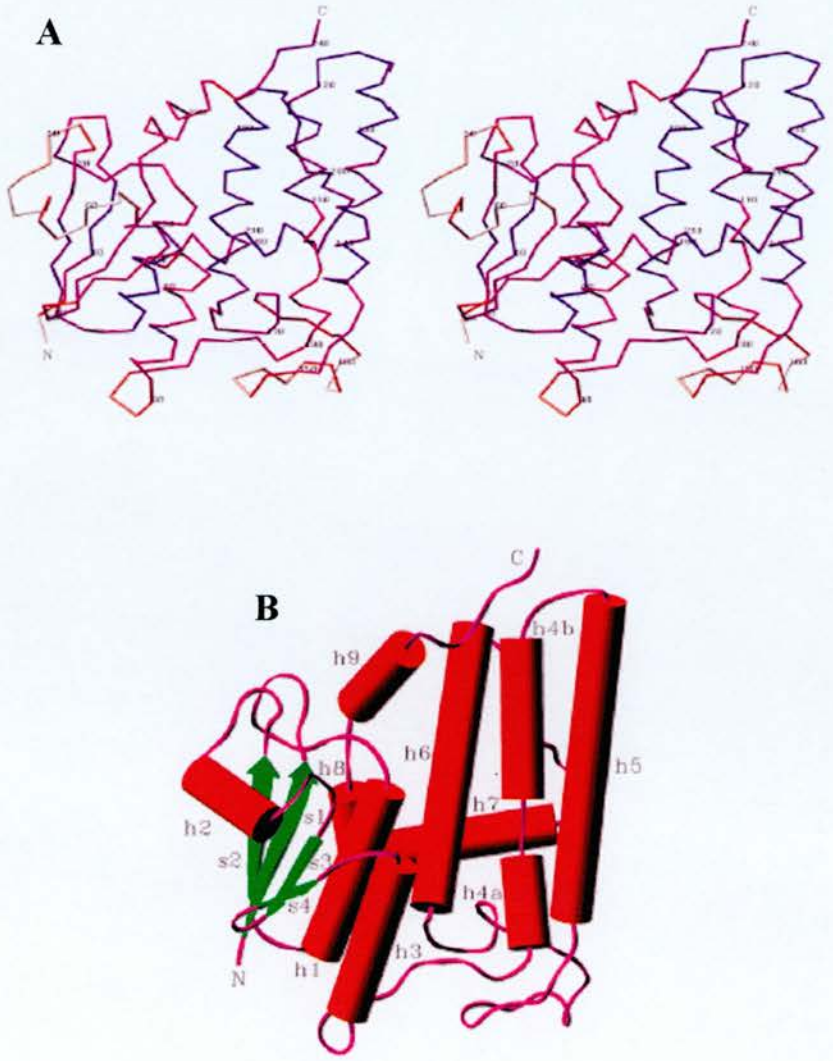


Figure 1-5. Crystal structure of the soluble form of CLIC1 at 1.4Å resolution.

Panel A: stereo diagram showing a C_α trace of CLIC1.

Panel B: schematic arrangements of secondary structural elements in CLIC1.

(Adapted from Harrop *et al.*, 2001)

1.5.4 CLIC3

CLIC3 was isolated using a yeast-two hybrid screen, in an attempt to identify proteins that associate with the *C* terminal tail of extracellular signal-regulated kinase 7 (ERK7; Qian *et al.*, 1999). ERK7 is a recently described member of the mitogen-activated protein (MAP) kinase family (Abe *et al.*, 1999). In contrast to other members of the ERK family (such as ERK1 and ERK2) the localisation, function and activity of ERK7 is regulated via protein interactions with its *C* terminus rather than extracellular signal-mediated activation.

CLIC3 cDNA encodes a 208 amino acid protein with a predicted molecular mass of 24 kDa. Like other members of the CLIC family, CLIC3 mRNA has a wide tissue distribution with high expression levels in human placenta, lung and heart, and lower expression levels in kidney, skeletal muscle and pancreas. A number of potential phosphorylation motifs for casein kinase II and protein kinase C, as well a potential *N*-myristylation site, have been identified in the CLIC3 sequence. Phosphorylation studies of CLIC3 indicated that although it is directly associated with the *C* terminus of ERK7, CLIC3 is not directly phosphorylated by ERK7. Immunofluorescence staining analysis of CLIC3 transfected cells showed the protein to be localised to the plasma and nuclear membrane as well to the cytoplasm. The association of CLIC3 with ERK7, in conjunction with the nuclear localisation of both proteins, suggest that CLIC3 may participate in cellular growth control. Further investigation of the function of CLIC3 using whole-cell patch clamp analysis from cells transfected with CLIC3 revealed that CLIC3 mediates Cl^- ion transport across the membrane of CLIC3 transfected cells (Qian *et al.*, 1999).

1.5.5 PARCHORIN (pp120)

Parchorin (pp120) was first identified as a phosphoprotein expressed in rabbit gastric glands (Urushidani *et al.*, 1987). It was shown that upon activation of acid secretion, parcachorin translocated from the cytoplasm, where it is mainly localised, to the apical membrane. As the protein was co-purified with a new type of protein kinase it was suggested that parchorin is a protein kinase itself (Urushidani *et al.*, 1999). Further studies on its kinase activity showed that it is rather a substrate for an unknown kinase (Nishizawa *et al.*, 2000).

Recently, the molecular and biochemical properties of parchorin were identified by (Nishizawa *et al.*, 2000). In this study the full length cDNA of parchorin was cloned, revealing a significant COOH terminal sequence homology with members of the CLIC family. Parchorin has an actual molecular mass of 65 kDa but because of its highly acidic nature it reduces the SDS / protein ratio and therefore migrates around 120 kDa as measured by SDS-PAGE. Parchorin, similar to other members of CLIC family, has a wide distribution in rabbit tissues: brain, chorioretinal epithelia, lacrimal glands, submandibular glands, airway epithelium, gastric mucosa and kidney. Functional studies on recombinant parchorin expressed in the LLC-PK1 kidney cell line revealed that it mediates a 50 % increase in Cl^- efflux when Cl^- is removed from the extracellular solution. However, this finding does not demonstrate whether parchorin is a Cl^- channel itself or acts as a regulator of a Cl^- channel.

1.5.6 CLIC5

CLIC5 was isolated from extracts of placental microvilli as a component of a multimeric complex interacting with the *C* terminus of a membrane cytoskeleton linking protein, ezrin (Berryman and Bretscher, 2000). However no evidence has been reported to prove direct binding of CLIC5 with the actin cytoskeleton. CLIC5 cDNA encodes a 251 amino acid protein with a predicted molecular mass of 28 kDa, and it has high amino acid homology (91 %) with the *C* terminus of bovine p64. Northern blot analysis of various human tissues revealed that CLIC5 mRNA is highly expressed in heart and skeletal muscle, and at lower levels in kidney, lung and placenta. Biochemical fractionation and immunofluorescence staining of recombinant CLIC5 showed that it is mainly localised at, or near the apical plasma membrane in polarised epithelial cells.

1.5.7 CLIC4 (p64H1)

Rat brain CLIC4 (originally named p64H1) was the first homologue of bovine kidney p64 (Landry *et al.*, 1993) to be identified (Howell *et al.*, 1996; Duncan *et al.*, 1997). Rat CLIC4 cDNA was cloned using a PCR-based approach and found to encode a microsomal membrane protein with a predicted M_r of 28,635 (Duncan *et al.*, 1997). *In situ* hybridisation histochemistry revealed a marked mRNA expression in rat hippocampus and cerebellum. Similar to other CLIC members, rCLIC4 mRNA has a wide tissue distribution (lung, liver, kidney, testis and skeletal muscle). Immunofluorescence analysis of cells expressing recombinant rCLIC4 showed that

the protein is localised in ER membranes (Duncan *et al.*, 1997) and in dense core vesicles (LDCVs; Chuang *et al.*, 1999).

The predicted sequence contained consensus phosphorylation sites for protein kinase C and protein kinase A. Furthermore, protein kinase C-mediated phosphorylation increased the apparent M_r of rCLIC4 to 43 kDa (Duncan *et al.*, 1997). In order to determine the membrane topology, intact microsomes containing recombinant rCLIC4 were treated with proteinase K revealing a large cytoplasmic domain, one membrane-spanning segment and a small *N*-terminal luminal domain (Duncan *et al.*, 1997; Figure 1-6).

Soon after the molecular cloning of CLIC4 from rat brain, the human, bovine and mouse versions were cloned, with 94 %, 95 %, and 97 % amino acid sequence identity to rat CLIC4 respectively (Chuang *et al.*, 1999; Edwards, 1999; Fernandez-Salas *et al.*, 1999).

The mouse homologue of CLIC4 (mtCLIC) was the first member of the CLIC family shown to be differentially regulated (Fernandez-Salas *et al.*, 1999). It was identified in an attempt to isolate potential p53 regulated genes expressed in differentiating keratinocytes from p53 +/+ and p53 -/- mice using PCR differential display. mtCLIC mRNA has a wide tissue distribution (heart, lung, liver and skin) in p53 +/+ and reduced levels in p53 -/- mice. Recombinant mtCLIC mRNA and protein expression levels were shown to be higher in p53 +/+ compared to p53 -/- transfected keratinocytes cells. In addition, expression levels were increased with $[Ca^{2+}]$ and tumor necrosis factor (TNF) - induced differentiation, independent of the genotype.

Immunofluorescence and subcellular fractionation studies of transfected keratinocytes revealed dual localisation of the recombinant and endogenous mtCLIC to mitochondrial fractions and to the cell cytoplasm. Like the rat and human CLIC4 homologues, mtCLIC has several putative protein motifs for cAMP-dependent protein kinase, protein kinase C, casein kinase II and tyrosine kinase mediated phosphorylation.

Early functional studies on rCLIC4 showed that when HEK-293 ER-vesicles containing recombinant rCLIC4 were reconstituted into planar lipid bilayers they gave rise to intermediate conductance, outwardly rectifying anion channels (Duncan *et al.*, 1997). However, as mentioned previously, it is not clear whether rCLIC4 is an ion channel itself or a channel regulator.

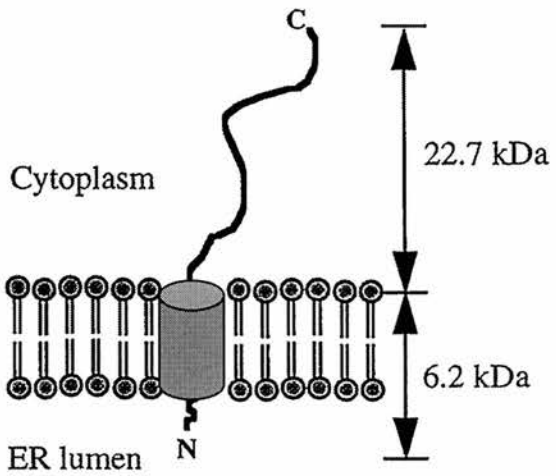


Figure 1-6. Membrane topology of rCLIC4.

rCLIC4 consists of a large cytoplasmic domain, one membrane-spanning segment and a small *N*-terminal luminal domain.
(Adapted from Duncan *et al.*, 1997)

1.6 AIMS

Over the past decade, an increasing number of researchers have been fascinated with the molecular and biochemical characterisation of intracellular ion channels and in particular with the most challenging field of anion channels. The chloride intracellular channel (CLIC) protein family is the most recently described family of putative anion channel proteins. A number of homologues have been identified since the discovery of the founder member, p64, including the first homologue rat brain CLIC4 (p64H1).

Although a number of reports have been published, the molecular basis of CLIC and p64-related proteins' cellular localisation and anion channel activity, are not yet clear. To help address these questions for rat brain CLIC4 (and possible other CLICs), a thorough investigation of the potential rCLIC4-associated proteins, its subcellular localisation and its electrophysiological characterisation were carried out. Pull-down and immunoprecipitation assays were set out to identify the proteins that interact with rCLIC4 in rat brain cytosol. rCLIC4 was over-expressed in cultured mammalian cells and its protein interactions were tested further. To investigate the channel activity of rCLIC4, purified recombinant protein and rCLIC4-proteoliposomes were incorporated into planar lipid bilayers.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS AND SUPPLIERS

2.1.1 GENERAL REAGENTS

All laboratory chemicals were of the highest possible grade and purchased from Sigma-Aldrich Company Ltd, (Dorset, UK), except where otherwise stated.

2.1.2 BACTERIAL STRAINS USED

XL1-Blue MRF': *Δ(mcrA) 183, Δ(mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac[F' proAB, lacI^{q}ZΔM15, Tn10].*

JM109: *endA1, recA1, gyrA96, thi, hsdR17, (r_k^-, m_k^+), relA1, supE44, Δ(lac-proAB), [F', tra D36, proAB, lacI^{q}ZΔM15].*

DH5α: F$^-$*φ80* d*lacZΔM15* Δ(*lac*ZYA-argF) *U169*, *endA1*, *recA1*, *hsdR17* (r_K^- m_K^+), *deoR*, *thi-1*, *supE44*, λ-*gyrA96*, *relA1*.

2.1.3 OTHER MOLECULAR BIOLOGY REAGENTS

Abcam Limited, Cambridge, UK

Covalight non-isotopic chemiluminescent reagent.

Amersham Pharmacia Biotech, UK.

pGEX-6P-1, pGEX-4T-1 vectors, PreScission Protease, Thrombin Protease, Glutathione 4B-Sepharose Fast Flow, dNTPs, Hybond-C pure nitrocellulose membrane, Hybond-P PVDF membrane, ECL detection kit.

Cytoskeleton Inc., Denver, USA.

Globular-actin (G-actin) β/ γ isoforms.

Boehringer Mannheim, Lewes, Sussex, UK.

Complete protease inhibitors cocktail, EDTA-free protease inhibitors cocktail.

GIBCO BRL, Life Technologies, Paisley, UK.

1kb DNA ladder.

Invitrogen Ltd, Paisley, UK.

Plasmid vector pcDNA3.1 $(+)/_{Zeo}$, Zeocin, Geneticin (G418), DH5α™ competent cells.

New England Biolabs, Hitchin, Hertfordshire, UK.

All restriction endonucleases.

Novagen, Beeston, Nottingham, UK.

Thrombin Cleavage Capture Kit.

Promega Corporation UK, Southampton, UK.

T4 DNA ligase, Taq DNA polymerase, Pfu DNA Polymerase, Proteinase K.

QIAGEN Ltd, Dorking, Surrey, UK.

Qiagen 500 plasmid purification kit, miniprep spin columns, QIAEX II DNA extraction kit.

Stratagene, La Jolla, CA, USA.

E. Coli strain XL1-Blue MRF'.

2.1.4 ANTIBODIES

Amersham Pharmacia Biotech, UK.

Goat Anti- GST, horseradish peroxidase conjugate.

Calbiochem, Beeston, Nottingham, UK.

Anti-mouse IgG (H&L), HRP-Linked Antibody.

Pierce & Warriner, Chester, UK.

Goat anti-rabbit IgG (H&L), horseradish peroxidase conjugate.

Transduction Laboratories, Lexigton, UK.

Caveolin-1, Rabbit Polyclonal, Caveolin-1, Mouse Monoclonal.

Upstate Biotechnology, Lake Placid, NY, USA.

Anti-Dynamin (Hudy1), mouse monoclonal IgG.

2.1.5 PHOSPHORYLATION REAGENTS

New England Biolabs, Hitchin, Hertfordshire, UK

Casein Kinase II, Casein Kinase II peptide substrate

Promega Corporation UK, Southampton, UK.

cAMP-dependant Protein Kinase (PKA) – catalytic subunit

Stratagene, La Jolla, CA, USA.

Protein Kinase C (PKC)

Amersham Pharmacia Biotech, Bucks, UK.

[γ-^{32}P] ATP

2.1.6 LIPIDS

AVANTI POLAR LIPIDS, Alabaster, (USA).

Sphingomyelin, Cholesterol, 1-palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphoethanolamine, 1,2-Diphytanoyl-Glycero-3-Phosphocholine, 1-palmitoyl-2-Oleoyl-sn-Glycero-3-[Phospho-L-Serine].

2.1.7 CULTURE MEDIA

Difco Laboratories, Surrey, UK.

All media components for bacterial cell culture

GIBCO BRL, Life Technologies, Paisley, UK.

All media for cell culture, Lipofectamine reagent.

2.2 CLONING IN PLASMID VECTORS

2.2.1 STANDARD RECOMBINANT DNA PROTOCOLS

Standard recombinant DNA protocols followed the procedures described by Sambrook *et al.*, (1989) including restriction enzyme digestion of DNA, ethanol precipitation of DNA, and DNA ligation.

2.2.2 GROWTH OF BACTERIAL CULTURE

Luria broth (LB) medium was prepared and autoclaved immediately to prevent any incidental bacterial growth from occurring. The medium was allowed to cool to room temperature and ampicillin was added to a final concentration 0.1 mg/ml. Using a sterile inoculating loop, a single colony was collected from an LB-Agar plate containing transfected *E.coli* expressing the GST fusion protein and transferred to a universal tube containing 5 ml LB medium. The inoculated culture was incubated on a shaker set at 250 rpm overnight at 37 °C. The overnight culture was diluted (1/100) in fresh LB medium containing ampicillin. The new cultures were incubated on a shaker at 37 °C (250 rpm) to an OD_{550} 0.6 to 0.8.

2.2.3 INDUCTION OF PROTEIN EXPRESSION AND RECOVERY OF BACTERIAL CELLS

Protein expression was induced by adding IPTG (to a final concentration 1.0 mM), followed by incubation at 37 °C for an additional 4 hrs. The cultures were then centrifuged for 15 min at 6,000 *g*, 4 °C. The pelleted cells were resuspended in PBS supplemented with anti-proteases (pancreas extract, pronase, thermolysin, chymotrypsin, papain). The cells were then lysed by probe sonication 8x 30 sec, with 1 min rests on ice between sonication (proteolysis was minimised by keeping the cells on ice throughout the sonication procedure). The lysate was centrifuged for 30 min at 13,000 *g*, 4 °C. A small sample (20 µl) was collected and analysed by SDS-PAGE and Western blotting.

2.2.4 PREPARATION OF COMPETENT BACTERIAL CELLS

200 ml of LB medium supplemented with 20 mM $MgSO_4$ was inoculated as described before. The cells were grown in a 1 L flask until the A_{600} reached 0.4-0.6. The cells were centrifuged at 4,500 *g* for 5 min at 4 °C. The pellets were gently resuspended in 0.4 of the original culture volume of ice-cold TFB1 (30 mM KAc, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl, 15 % (v/v) glycerol, pH 6.8) and incubated on ice for 5 min. The cells were further pelleted by centrifugation as before and were gently resuspended in 1/25 of the original culture volume of ice-cold TFB2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15 % (v/v) glycerol, pH 6.5). The cells were incubated on ice for 15-60 min, quick-freezed in dry ice/ isopropanol bath in 200 µl aliquots, and kept at -70 °C for up to six months.

2.2.5 TRANSFORMATION OF COMPETENT BACTERIAL CELLS

Aliquots of 200 μl frozen, bacterial competent cells was thawed on ice for 30-45 min. 10 ng of plasmid DNA were added to the tube, mixed by gently swirling the pipette tip, and incubated on ice for 30 min. The tube was heated to 42 °C in a water bath for 1 min, and then placed on ice for 2 min to cool. LB medium (~ 800 μl) was added to the tube and incubated for 45 min at 37 °C in a shaking incubator (200 rpm). An appropriate dilution of the mixture (usually 50 μl and 100 μl) was added to LB medium plates containing selection antibiotics. The medium was spread evenly on the surface of the agar by using a sterile glass rod until it was almost absorbed by the agar. The plate was inverted and incubated at 37 °C for 12-16 hrs until the colonies appeared.

2.2.6 PURIFICATION OF DNA FRAGMENTS

2.2.6.1 SMALL SCALE PLASMID PREPARATION (MINI-PREP)

In this method a QIAprep Spin miniprep kit (Qiagen) was used, according to the manufacturer's manual. This method used an alkaline solution to lyse the bacteria. Instead of the phenol/ chloroform extraction and ethanol precipitation of DNA, this kit included a silica-gel membrane in a spin column for DNA binding, and DNAs were eluted by distilled water.

2.2.6.2 LARGE SCALE PLASMID PREPARATION (MAXI-PREP)

The QIA filter plasmid maxi kit (Qiagen) was used for large-scale plasmid preparation, according to the manufacturer's manual. This method was based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins, and low-molecular-weight impurities were removed by a high salt wash. Plasmid DNA was eluted in a high-salt buffer, and then concentrated and desalted by isopropanol precipitation.

2.2.7 DNA GEL ELECTROPHORESIS

As described by Sambrook *et al.* (1989, 6.1-6.62) an agarose gel (1 % w/v) was used for separating DNA fragments of different lengths. Gel electrophoresis was performed in a buffer containing 45 mM Tris-borate and 1 mM EDTA (TBE). The negatively charged DNA moved from cathode to anode and was visualised under ultraviolet (UV) light by adding ethidium bromide solution during the preparation of the gel.

2.2.8 PURIFICATION OF DNA FRAGMENTS

DNA was digested by appropriate restriction endonucleases (New England Biolabs), and loaded on agarose gels (1 % (w/v) unless otherwise stated) to separate any undigested circular DNA. When each DNA band was separated clearly, the

desired fragments were cut out and purified using a QIAEX II gel extraction kit (Qiagen), according to the manufacturer's manual. This method used silica-gel particles for binding DNAs. The gels were washed before DNA were eluted by distilled water.

2.2.9 GENERATION OF FLAG-TAGGED CLIC4 BY INVERSE PCR MUTAGENESIS

Using Inverse Polymerase Chain Reaction Mutagenesis (IPCRM) the Flag epitope sequence (DYKDDDDK) was fused in-frame with the carboxyl terminus of CLIC4, which was already subcloned into pcDNA 3.1(+)/ zeo vector (Invitrogen). The method followed was exactly as described before by Gama and Breitwieser, (1999).

2.3 PROTEIN PROTOCOLS

2.3.1 SODIUM DODECYLSULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS- PAGE)

Protein electrophoresis followed the method of Laemmli (1970). The SDS-polyacrylamide gels were poured in a Mini-Protean II system (Bio-Rad), which was assembled according to the manufacturer's instructions. The solutions for the separating gels (total volume 10 ml) were prepared in the order as shown in Table 2-1. The separating gels were mixed well and then poured between the glass plates of the gel apparatus and overlaid with water. After the gels had polymerised (about 20

min), the overlaid water was poured out and the gels were washed three times with water. A 3.5 % (w/v) of stacking gel (total volume 5 ml) was prepared and poured over the separating gel (Table 2-2). Before the stacking gels polymerised (about 15 min), a comb was inserted to allow the wells to form. The samples were denatured by heating to 100 °C for 5 min in an equal volume of 2 X SDS gel sample buffer (2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 10 % (v/v) glycerol, 62.5 mM Tris-HCl, pH 6.8), and then placed on ice. Once the stacking gels had polymerised, the comb was carefully removed and the wells were washed with water to remove unpolymerised acrylamide. The protein samples were loaded and voltage applied to the gel at 8 V/cm until the dye front moved into the stacking gel, and then 15 V/cm until the dye front reached the bottom of the separating gel. Proteins moved from cathode to anode.

2.3.2 STAINING AND DESTAINING OF PROTEIN GELS

To visualise the proteins the SDS-polyacrylamide gels were stained in a solution containing 0.25 % (w/v) Coomassie Brilliant Blue R250, 90 % (v/v) methanol:H_2O (1:1 v/v), and 10 % (v/v) glacial acetic acid for 30 min at room temperature. The gels were then destained by immersing them in 90 % (v/v) methanol: H_2O (1:1 v/v) and 10 % (v/v) glacial acetic acid for 24 hr, changing the destaining solution twice. To make a permanent record, the gels were dried on a vacuum gel drier (Hoefer), according to the manufacturer's manual.

Component	Volume of components for different percentages of acrylamide / ml			
	8 %	10 %	12.5 %	15 %
H_2O	4.03	3.53	2.905	2.28
40 % (w/v) acrylamide/bisacrylamide	2.00	2.50	3.125	3.75
1 M Tris-HCl, pH 8.8	3.76	3.76	3.76	3.76
10 % (w/v) SDS	0.10	0.10	0.10	0.10
10 % (w/v) Ammonium Persulphate	0.10	0.10	0.10	0.10
TEMED	0.01	0.01	0.01	0.01

Table 2-1 . Composition of separating (resolving) gels in SDS-PAGE.

Component	Volume of components added / ml
H_2O	3.20
40 % (w/v) acrylamide/bisacrylamide	1.25
0.5 M Tris-HCl, pH 6.8	0.44
10 % (w/v) SDS	0.05
10 % (w/v) Ammonium Persulphate	0.05
TEMED	0.005

Table 2-2. Composition of stacking gel in SDS-PAGE.

2.3.3 IMMUNOBLOTTING AND ANTIBODY PRODUCTION

Proteins separated by SDS-PAGE were transferred onto pre-soaked Hybond C-Pure nylon membranes (Amersham) or PVDF membrane using a wet transfer apparatus (Hoefer) at a constant current of 400 mA for 70 min. (Transfer buffer: 25 mM Tris-HCl, 190 mM glycine, 20 % (v/v) methanol). Following the transfer of proteins to the membranes, the non-specific binding sites on the membrane were blocked by incubation in PBS, 0.05 % (v/v) Tween 20 (PBS-T), containing 5 % (w/v) dried-milk (Marvel) or 3 % (w/v) bovine serum albumin (BSA) for at least 1 hour.

A rabbit polyclonal antiserum was raised by Scottish Antibody Production Unit (SAPU) against recombinant affinity-purified CLIC4 cytoplasmic domain emulsified with TiterMax (CytRx). Polyclonal CLIC4 antibody (1:5000 dilution in PBS-T) or affinity purified anti-CLIC4 antibody (1:500 dilution in PBS-T) were incubated with the membrane for 2 hr at room temperature. The membranes were washed 4 times for 5 min, with gentle agitation, in PBS, and then incubated for 1 hr at room temperature with goat anti-rabbit IgG-horseradish peroxidase conjugate (1:2000 dilution in blocking buffer). After 1 hr incubation, the membranes were washed 4 times for 5 min, with gentle agitation, in PBS-T. Signals were detected using the ECL (enhanced-chemiluminescence; Amersham) method, followed by exposure to photographic film.

2.3.4 AFFINITY PURIFICATION OF ANTIBODIES FROM CRUDE SERUM

Anti-CLIC4 immune serum was passed through a Sepharose 4B column conjugated with GST protein to remove cross-reactivities. The final flow-through was affinity purified by a GST-CLIC4 Sepharose column, eluted with 0.2 M Glycine pH 2.8 and neutralised with 0.1 M Tris-HCl, pH 8.5. To remove any further non-specific cross-reactivities, anti-CLIC4 immune serum was purified on immunoblots as described by Olmsted, (1981). Recombinant, purified rCLIC4 was electrophoresed loaded in a 10 % (w/v) SDS-polyacrylamide gel and the protein was transferred onto PVDF membranes as described before. The blots were then stained with Ponceau-S solution and the bands corresponding to rCLIC4 (~30 kDa) were located and cut in horizontal strips (usually 0.2-0.3 cm wide). The blots were blocked with PBS-dried milk for 1 hr, washed with PBS-T and incubated with anti-CLIC4 for 12-16 hr at 4 ^{o}C. After washing with PBS-T, the bound antibody was eluted with 0.5 ml of 0.2 M glycine (pH 2.8) for 20 min at 4 ^{o}C. The elution buffer was collected and immediately neutralised with 0.1 M Tris-HCl (pH 8.5). The purified antibody was dialysed in PBS, concentrated (up to ten fold) and kept at 4 ^{o}C for up to four weeks.

2.3.5 AFFINITY CHROMATOGRAPHY PURIFICATION OF GST-CLIC4

Soluble GST fusion proteins were purified from cell lysate supernatants by affinity chromatography on a Glutathione Sepharose 4B column according to the manufacturer instructions. Glutathione Sepharose 4B resin was prepared as a 50 % (v/v) slurry in PBS (1 ml of per L of culture) prior to sample loading. The sample

slurry was incubated overnight on a platform shaker at 4 °C. A small fraction (20 µl) was collected for analysis by SDS-PAGE to verify that the fusion protein was bound and that column capacity was not exceeded. The column was washed with 5 to 10 bed volumes of PBS to remove any serine protease inhibitors before the cleavage.

2.3.6 PROTEASE CLEAVAGE OF GST FUSION PROTEINS IN SOLUTION

The GST affinity tag was removed by cleaving with thrombin or precision proteases. The protease was added to the column (5 U per one ml resin bed volume). The column was incubated for 2 hrs at room temperature (or overnight at 4 °C) on a rotor. Cleaved CLIC4 was eluted from the column and stored at 4 °C (up to one week) until needed. GST bound on the glutathione beads was eluted with glutathione elution buffer (1 ml elution buffer per 1 ml resin bed volume). Small fractions were collected from each step (20 µl) for analysis by SDS-PAGE and Western blotting. The yield of fusion protein, GST and CLIC4 was monitored by protein estimation using the Lowry method (Markwell et al., 1981).

2.3.7 PREPARATION OF MICROSOMAL MEMBRANES

The method followed the procedure described by Yoneshima et al. (1997) with some modifications. Two T75 culture flasks of transfected cells were homogenised in 3 ml homogenisation buffer (0.32 M sucrose, 5 mM Tris-HCl, pH 7.4, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 1 µg/ml leupeptin,

0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 150 μg/ml benzamidine). After 30 strokes using a tight-fitting glass-teflon homogeniser, the homogenate was centrifuged at 1,000 *g* for 10 min at 4 °C. The supernatant was re-centrifuged at 100,000 rpm in a TLA-100.3 rotor using a TL-100 centrifuge (Beckman) for 45 min at 4 °C. The pellet was resuspended in 80 μl homogenisation buffer to be used immediately or quick frozen in liquid nitrogen and stored at -70 °C.

2.3.8 ANALYSIS OF PROTEINS BY IMMUNOPRECIPITATION

Immunoprecipitation technique is a very simple method, but it takes time and accuracy to find the optimal working conditions. Almost every antigen requires a particular protocol treatment. The following, are two methods that have been used for CLIC4 in eukaryotic cell extract and rat brain cytosol.

2.3.8.1 IMMUNOPRECIPITATION FROM EUKARYOTIC CELL EXTRACTS

HEK-293 cells were grown to subconfluence, in two T175 culture flasks. The media was aspirated and cells were washed with ice-cold PBS twice. One ml of ice-cold IP buffer (10 % (v/v) glycerol, 100 mM NaCl, 2 mM EGTA, 5 mM molybdate, 50 mM Tris pH 7.5, anti-protease cocktail and phosphatase inhibitors: 2 mM Na Orthovanadate, 1 mM NaF) was added per flask. The cells were harvested using a cell scraper and then passed twice through a 21 g syringe to lyse them and to shear DNA. The cell lysates were centrifuged first at 10,000 *g* for 10 min at 4 °C to remove

particulate material and the supernatant was re-centrifuged at 100,000 *g* for 20 min at 4 °C. One ml of the supernatant was precleared with non-immune serum and protein G-Sepharose for 1 hr at 4 °C. After a 5 min spin (1000 *g*) to concentrate the beads, the supernatant was incubated with 12 μg/ml of rabbit polyclonal CLIC4 antibody or 10 μg/ml mouse monoclonal M_2 Flag antibody at 4 °C for 2 hr. The rCLIC4-protein complex was immunoprecipitated overnight by protein G- Sepharose (1:1 in PBS) at 4 °C. Then the beads were spun down five times as before, washed with ice-cold PBS each time, and eluted with 40 μl of 2x SDS-loading buffer. The immunoprecipitated proteins were analysed by SDS-PAGE and Western blotting.

2.3.8.2 IMMUNOPRECIPITATION FROM RAT BRAIN CYTOSOL

Rat brain cytosol (1 ml) was precleared by incubation with protein G-Sepharose (1:1 in PBS) at 4 °C for 1 hr. The precleared lysate was then incubated with anti-CLIC4 polyclonal antibody (12 μg/ml), for 2 hr at 4 °C. The rCLIC4-protein complex was immunoprecipitated overnight by protein G- Sepharose (1:1 in PBS) at 4 °C. The beads were washed thoroughly with ice-cold PBS, heated briefly with 40 μl 2x sample buffer and the eluted proteins were analysed by SDS-PAGE and Western blotting.

2.4 CELL CULTURE PROTOCOLS

2.4.1 MAMMALIAN CELL CULTURE

HEK-293 cells (a transformed human embryonic kidney fibroblast cell line; Graham et al., 1977) and MDCK (a transformed human embryonic kidney cell line; Gaush et al., 1966) were cultured in DMEM (Gibco BRL) supplemented with 10 % (v/v) foetal calf serum (Gibco BRL). Cells were cultured and passaged according to standard procedures (Doyle and Ruegg, 1985).

2.4.2 TRANSFECTION OF MAMMALIAN CELLS

Transfection was performed by using Lipofectamine reagent (Gibco BRL) and the manufacturer's instructions were followed precisely.

2.4.3 INDIRECT IMMUNOFLUORESCENCE

HEK-293 and MDCK cells were cultured in four-well tissue culture plates containing 13 mm^2 sterile glass coverslips (BDH). After 48 hr of transfection, the culture medium was removed by aspiration. The cells were washed 3 times in complete PBS (PBS supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+}, pH 7.4). The cells were then fixed by incubation with 1 ml per well of 4 % (w/v) buffered paraformaldehyde (in PBS, pH 7.0) at room temperature for 10 min. After the incubation, the solution was removed by aspiration, and the fixed cells were washed 3 times in complete PBS. The cells were then incubated in 1 ml of 50 mM NH_4Cl in PBS for 10 min to quench potential auto fluorescence. The cells were again washed 3

times in PBS, and permeabilised in 1 ml per well of 0.1 % (v/v) Triton X-100 in PBS for 5 min. After 3 washes in PBS, the coverslips were inverted onto 100 µl of antibody (diluted 1:100 in 0.2 % (v/v) fish skin gelatin in PBS) on a piece of parafilm and incubated for 1 hr at room temperature. The coverslips were washed 3 times in 0.2 % (v/v) fish skin gelatin/PBS for over 5 min, followed by 3 washes in PBS. The coverslips were then inverted onto 100 µl of fluorochrome-labelled secondary antibody (diluted in 0.2 % (v/v) fish skin gelatin/PBS), incubated at room temperature for a further hour followed by washing 3 times in 0.2 % (v/v) fish skin gelatin/PBS and 3 times in PBS. The coverslips were mounted onto slides using 20 µl of Aquamount (BDH) for 2 hr before examination. Confocal microscopy was carried out using a Leica Confocal fluorescence microscope. The laser output and the detection gain were adjusted to avoid saturation of the signal.

2.5 OTHER PROTOCOLS

2.5.1 CAVEOLAE ISOLATION BY SUCROSE DENSITY CENTRIFUGATION

Caveolin-enriched membranes were isolated as described previously by Pike and Casey (1996) with minor modifications. HEK-293 or MDCK cells were plated in T-175 flasks until confluence was reached. Monolayers of the cells were washed twice in ice-cold PBS, incubated on ice for 15 min, with 1 ml lysis buffer (25 mM MES; pH 6.5, 150 mM NaCl, 1 % (v/v) Triton X-100, 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride). One ml of the cell lysate was then mixed with 1 ml of 25 mM MES; pH 6.5, 150 mM NaCl, 1 mM EGTA, 80 % (w/v) sucrose, by

passing the sample through a 24-gauge needle five times. The sample was layered on top with 6 mls of 25 mM MES; pH 6.5, 150 mM NaCl, 1 mM EGTA, 30 % (w/v) sucrose and 4 mls of 25 mM MES; pH 6.5, 150 mM NaCl, 1 mM EGTA, 5 % (w/v) sucrose, in a 12 ml polycarbonate centrifuge tube (Beckman). The tubes were centrifuged for 3 h at 4°C at 175,000 *g* in an SW41 rotor (Beckman). Fractions of 1 ml were collected from the top of the gradient and the pellet was resuspended in 1 ml PBS. 15 µl of each fraction were mixed with equal volume of 2x SDS-loading buffer, run in a 12.5 % (w/v) polyacrylamide gel and analysed by western blot as previously described.

2.5.2 CLIC4 BINDING ASSAY

CLIC4 - cytoplasmic domain (Cyt. D.) and 14-3-3ζ proteins were expressed as GST fusion proteins as previously described. GST 14-3-3ζ (1 mg, 16 µM) coupled to glutathione beads, was incubated with 300 µg (13 µM) of thrombin-cleaved CLIC4 (Cyt. D.) overnight in 1 ml reaction mixture. The beads were placed in a column and unbound proteins were washed away with 100 ml of binding buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 % (v/v) glycerol). In some cases the last 10 ml wash were collected. Non-specific, bound proteins were eluted with 10 ml of binding buffer supplemented with 2 M NaCl (final concentration). The eluate was concentrated down to 65 µl using Centricon (Amicon) concentrators, mixed with an equal volume of sample buffer and separated

on a 10 % (w/v) polyacrylamide gel. The bands that corresponded to bound CLIC4 were detected by anti-CLIC4 polyclonal antibody, on a Western blot.

2.5.3 PEPTIDE COMPETITION ASSAY

For the peptide competition assays, the binding assay was repeated as described in section 2.4.2. This time the binding of CLIC4 (cyt. D.) to the immobilised GST 14-3-3 was competed in the presence of a ten fold molar excess (approximately 0.3 mg) of either phosphorylated or non-phosphorylated forms of two synthetic peptides. The peptides correspond to the 14-3-3 binding sequence LSGRGRST**S**TPNVHMV of Raf peptide, where the underlined serine residue was either phosphorylated or non-phosphorylated. rCLIC4 binding was further investigated by the addition of a ten fold molar excess of an unrelated control peptide NVVGARRSSWRVISSIEQKT (14-3-3γ isoform, residues 51-70). For quantitative analysis, proteins bound to the immobilised GST 14-3-3 were washed extensively with 100 ml washing buffer and elution of the proteins was achieved by boiling the GS4B beads in an equal volume of sample buffer. Equal volumes of samples were run on a 10 % (w/v) polyacrylamide gel and analysed by Western blotting with the anti-CLIC4 polyclonal antibody.

2.5.4 ACTIN SPIN-DOWN ASSAY

Lyophilised G-actin (a mixture of β and γ isoforms; Cytoskeleton Inc.) was resuspended in 100 µl pure ddH_2O according to the manufacturer's instructions. Equimolar amounts (5 mM) of G-actin and rCLIC4 (full length or cytoplasmic domain) or cofilin (a kind gift from Dr S. Maciver) solutions were mixed in 500 µl reaction mixture, containing polymerising buffer (50 mM KCl, 0.2 mM ATP, 0.5 mM DTT and 0.2 mM $CaCl_2$, 1 mM EGTA and 1 mM $MgCl_2$). The solutions were buffered to pH 6.5 with 10 mM imidazole-HCl, or to pH 8.0 with 10 mM Tris-HCl. Following a 60 min incubation step at room temperature, actin was polymerised (F-actin) and separated from the non-polymerised actin (G-actin) by centrifugation (300,000 *g* for 20 min in a Beckman TL-100.2 rotor). The supernatant, containing the G-actin, was removed and mixed with an equal volume of 2x SDS-PAGE loading buffer. After the addition of 500 µl of polymerisation buffer, the pellets were incubated for 10 min at room temperature and then mixed with equal volume of 2x SDS-PAGE loading buffer. Equal volumes of the pellet and supernatant fraction were analysed on a 12.5 % (w/v) SDS-polyacrylamide gel and the bands were visualised after staining with Coomassie Brilliant Blue stain.

2.5.5 PROTEIN PHOSPHORYLATION PROTOCOLS

2.5.5.1 *IN VITRO* PHOSPHORYLATION ASSAYS

Recombinant GST-rCLIC4 was purified on GS4B column and the GST-moiety was cleaved as described earlier. Cleaved rCLIC4 was used as substrate for protein kinase C- (Stratagene), protein kinase A- (Promega) and casein kinase II-

(New England Biolabs) mediated phosphorylation. The reaction mixtures for each kinase are summarised in Table 2-3. 10 μCi (1 μl) of [γ-^{32}P] ATP (Amersham) was added per reaction and the mixtures were incubated at 30 °C for 10 min. The reactions were terminated by the addition of SDS-loading buffer. The samples were then analysed by SDS-PAGE (Novex 12 (w/v) % Tris gels). The gels were allowed to fully dry, placed in an intensifying screen cassette and exposed to ^{32}P sensitive film (1 hr at 70 °C – 36 hr at room temperature).

Component	PKA	PKC	CK II
Cold ATP (10 mM)	3 μl	3 μl	3 μl
Protein (0.5 μg/μl)	5 μl	5 μl	5 μl
Phospholipids (diolein and PC)	-	3 μl	-
Sterile Water (up to 30 μl)	17 μl	14 μl	17 μl
10x Reaction Buffer	3 μl	3 μl	3 μl

Table 2-3 Components of phosphorylation reactions.

10x Reaction Buffers: protein kinase A (PKA) - 20 mM $MgCl_2$, 40 mM Tris HCl, pH 7.4; protein kinase C (PKC) - 100 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM EGTA, 400 mM MES, pH 6.0; casein kinase II (CKII) – 100 mM $MgCl_2$, 500 mM KCl, 200 mM Tris HCl, pH 7.5.

2.5.5.2 ACTIVATION OF SODIUM ORTHOVANADATE

To achieve maximal inhibition of protein phosphotyrosyl-phosphatases sodium orthovanadate was dissolved in dH_20 (100 mM stock) and the pH was adjusted to 10.0 with HCl (yellow colour; Gordon, 1991). The solution was placed in a boiling water bath for 5 min and then placed on ice to cool. At the cooling stage the pH was much lower than 10.0 and adjusted back with NaOH. The boiling and cooling steps were repeated till the yellow colour was gone and pH was stabilised at 10.0. Sodium orthovanadate was stored in aliquots at -20 °C.

2.5.5.3 DETECTION OF TYROSINE PHOSPHORYLATED PROTEINS

Transfected and control HEK-293 cells were cultured in T75 flasks until they reach 80-90 % confluency. Pervanadate treatment of the cells was carried out as described previously (Lyons et al., 2001). HEK-293 cells were treated for 30 min with 0.1 mM pervanadate (20 µl of a fresh solution containing 50 mM sodium Orthovanadate and 50 mM H_2O_2 was added to 10 ml of culture medium). The media were aspirated and cells were washed with ice-cold PBS twice. One ml of IP buffer (section 2.3.8.1) was added per flask. Solubilised cells were scraped using a cell scraper and the collected lysate was passed twenty times through a 21 g syringe to homogenise the cells and shear the DNA. The lysate was then incubated on ice for 30-45 min followed by centrifugation first at 10,000 *g* for 10 min at 4 °C to remove particulate material and then at 300,000 *g* for 20 min at 4 °C. The resulting supernatants were immunoprecipitated with anti-CLIC4 immune serum as described previously and analysed by SDS-PAGE and anti-phosphotyrosine immunoblotting.

2.5.6 PLANAR LIPID BILAYER RECONSTITUTION

2.5.6.1 FORMATION OF LIPOSOMES AND ESTIMATION OF THE TRAPPED VOLUME

Unilamellar liposomes and proteoliposomes were prepared by detergent dilution or detergent dialysis, using n-octylglucoside, cholate and CHAPS. Briefly, lipid and protein suspensions were solubilised in a detergent solution containing (150 mM KCl, 20 mM Tris-HCl; pH 7.4, 1 % (w/v) n-OG, 1 % (w/v) cholate or 1.4 % (w/v) CHAPS). The detergent was removed either by gel filtration using Sephadex G25 (fine grade) or by dialysis for at least 36 hours against 150 mM KCl and Tris-HCl pH 7.4 (3 changes). In order to estimate the capture volume, liposomes were prepared as before in 100 mM Na chromate (Hope et al., 1986). Following gel filtration to remove the external chromate, 500 µl aliquots were collected. The trapped chromate was determined by solubilising the liposomes aliquots in 10 mM Triton X-100, followed by measuring the absorbance at 380 nm (A_{380}) for chromate. The trapped volume was expressed as the percentage difference of chromate absorbance in Triton X-100 treated aliquots and the original absorbance of chromate in solution.

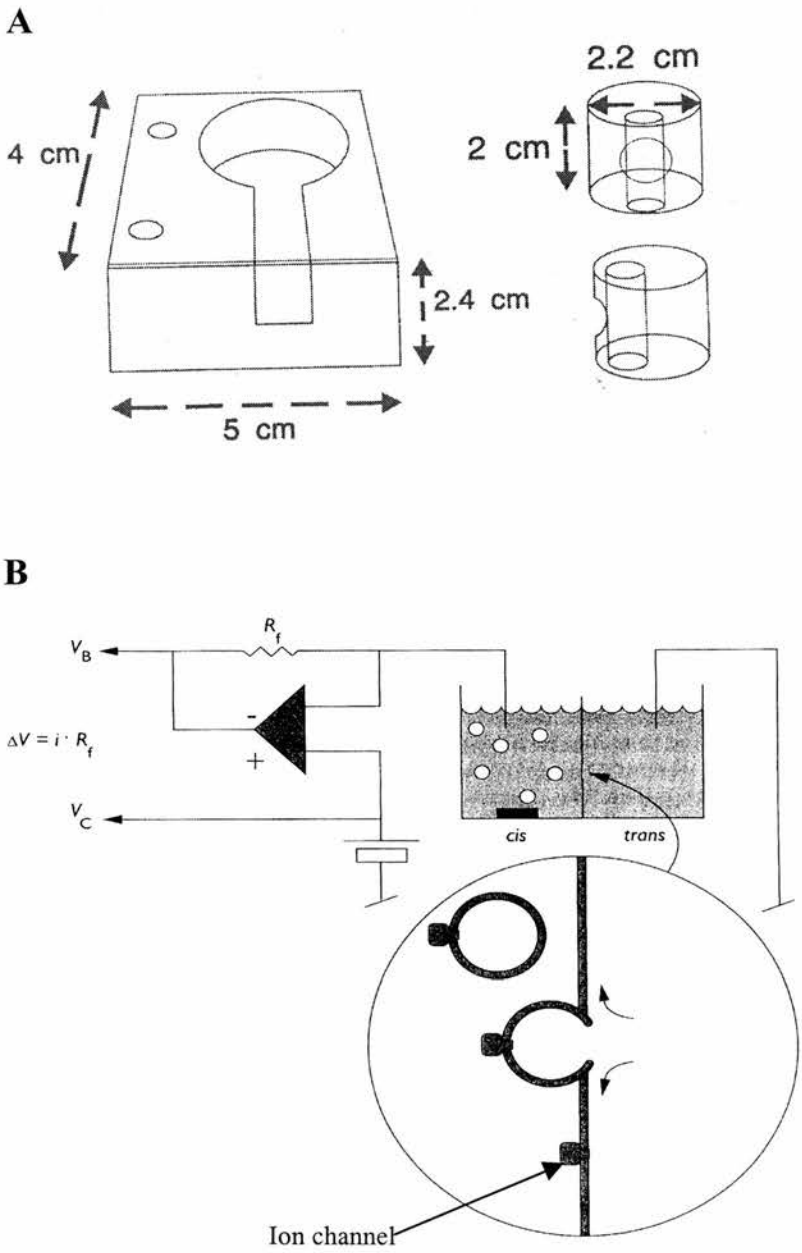


Figure 2-1 Reconstitution of CLIC4 containing liposomes in the bilayer set up.

Panel A. Experimental blocks (*cis* and *trans* chambers) for the formation of bilayers. Adapted from Williams (1995).

Panel B. Details of the electric circuit used in the bilayer set up (top) and the fusion of a single vesicle to the bilayer (bottom). Adapted from Ion Channels: A Practical Approach (Ashley, 1995).

2.5.6.2 BILAYER SYSTEM

The bilayer set-up is similar to those described by Williams (1995). Lipid bilayers were prepared using palmitoyl-oleoyl phosphoethanolamine (POPE): palmitoyl-oleoyl phosphoserine (POPS) (1:1) dispersed in chloroform. 7.5 µl each of 50 mg/ml POPE and POPS prepared in chloroform was mixed, and the chloroform was evaporated under a stream of nitrogen. 25 µl of n-decane was then added to the dried phospholipids to give a final phospholipid concentration of 30 mg/ml. A small amount of lipid dispersion (~ 1 µl) was applied using a flexible plastic stick around the 0.3 mm hole in a polystyrene partition separating two solution-filled chambers (~ 600 µl), designated *cis* and *trans* (Figure 2-1). Bilayers formed spontaneously as observed by monitoring the increase in membrane capacitance, which accompanied thinning. The *cis* side of the bilayer was voltage-clamped at potentials relative to the *trans* side using a patch clamp amplifier. The potential applied across the bilayer was termed the *holding potential* or *voltage - clamp potential.* Transmembrane currents were low-pass filtered and digitally recorded. All the bilayers used had a conductance of < 10 pS and a capacitance of ~500 pF. To incorporate channels, purified detergent-solubilised recombinant rCLIC4, or rCLIC4 reconstituted into liposomes were added to the *cis* chamber to a final concentration of 1 ng/ml, in the presence of a salt gradient (usually *cis* 300 mM KCl; *trans* 50 mM KCl buffered with 20 mM Tris HCl pH 7.4). The solutions bathing the bilayer were changed according the presence or lack of channel activity. Channel recordings were post-filtered to reduce high frequency noise and analysed using the software Axotape2 (Axon Instrument).

CHAPTER 3

SUBCELLULAR LOCALISATION OF rCLIC4

3.1 GENERATION OF FLAG-TAGGED rCLIC4 BY INVERSE PCR MUTAGENESIS

3.1.1 INTRODUCTION

The polymerase chain reaction (PCR) has been extensively used to amplify a DNA sequence using a pair of oligonucleotide primers, each complementary to one end of the DNA target sequence. In theory any DNA molecule can be amplified, providing appropriate primers can be designed. This is carried out in a three-step reaction cycle (denaturation, primer annealing and polymerisation), whereby the oligonucleotide primers extent towards each other by a thermostable DNA polymerase enzyme. Briefly the reaction cycle comprises a 95 °C step to denature the target DNA (denaturation step), followed by the binding of the primers to the template at ~55 °C (annealing step) and a 72 °C extension of the primers (polymerisation step). Magnesium (Mg^{2+}) and dNTPs are required in addition to template, primers, buffer and DNA polymerase enzyme. PCR primers need to be about 18-30 nucleotides long and have a similar *G+C* content so that they can anneal to their complementary sequences at similar temperatures.

To permit the insertion of the Flag epitope sequence (DYKDDDDK) in-frame with the carboxyl terminus of rCLIC4 cDNA a modified protocol of the Inverse PCR mutagenesis (Gama and Breitwieser, 1999) has been used. rCLIC4 cDNA was subcloned into pcDNA3.1 /zeo (5' *Bam*H1 – *Xho*I 3' restriction fragment) containing the Kozak consensus sequence (A/GCTATG; Kozak, 1987a; 1987b) for the initiation of transcription (Figure 3-1). pcDNA3.1 /zeo is a mammalian expression vector that contains the cytomegalovirus (CMV) enhancer

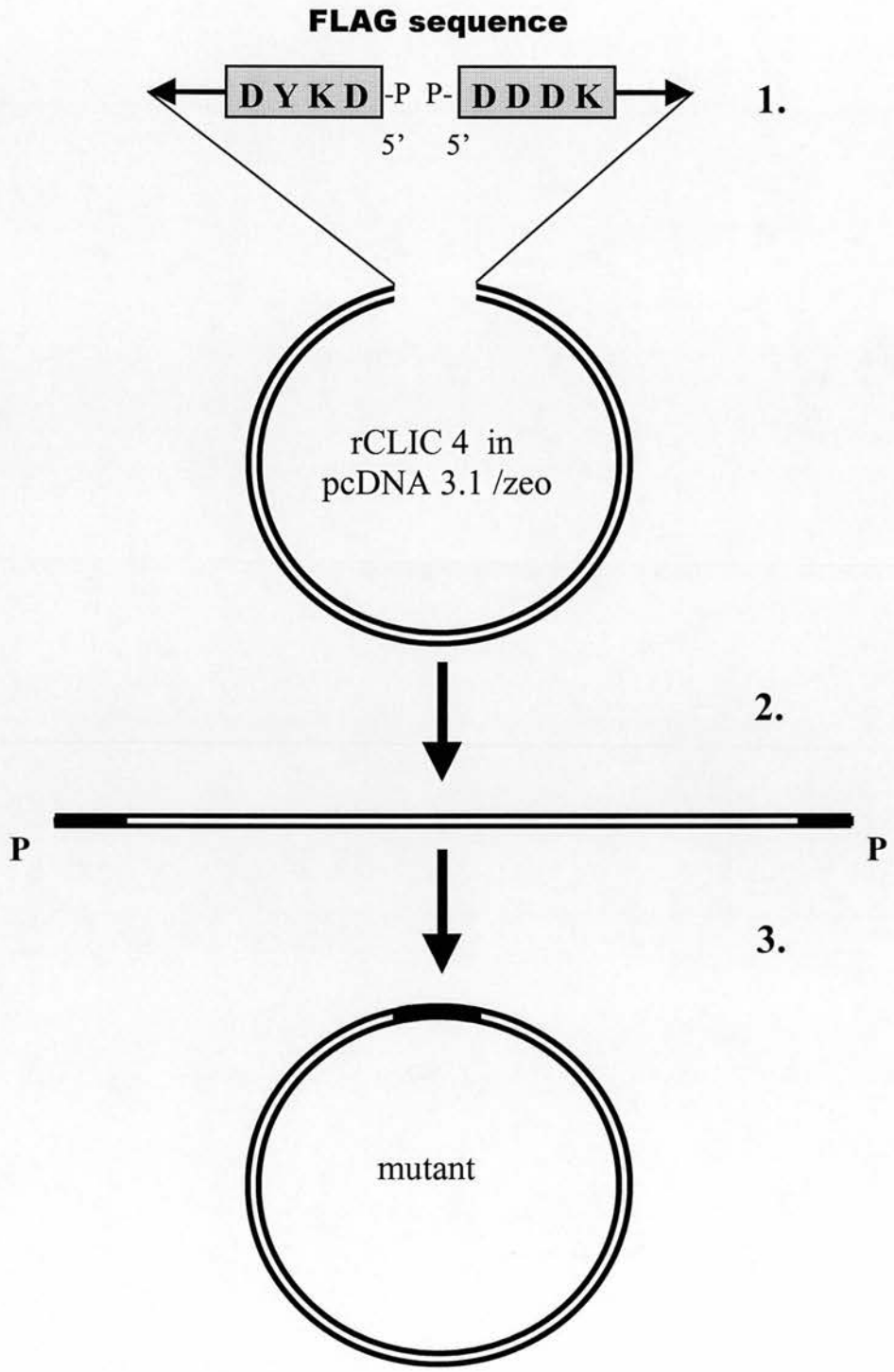


Figure 3-1. Generation of rCLIC4-Flag construct.

1. Design of adjacent, non-overlapping phosphorylated primers containing the in-frame FLAG sequence; **2**. Inverse PCR with *Pfu* DNA polymerase; **3**. Ligation followed by digestion with *DpnI* and transformation into bacteria cells (Gama L and Breitwieser G., 1999).

promoter (for high level expression; Anderson et. al., 1989), bovine growth hormone (BGH) polyadenylation signal and transcription termination sequence (to enhance mRNA stability; Goodwin and Rottman, 1992), the SV40 origin (for episomal replication and simple vector rescue in cell lines expressing the large T antigen), a T7 RNA polymerase promoter and the Zeocin gene (for selection of stable cell lines).

3.1.2 GENERATION OF FLAG TAGGED rCLIC4

For the insertion of the epitope, a pair of adjacent, non-overlapping primers were designed, both phosphorylated at the 5' end. The upstream primer consisted of 20 nucleotides complementary to the end of rCLIC4 DNA without the stop codon, plus 12 in-frame nucleotides corresponding to the first half of the Flag DNA sequence. The downstream primer contained the other half of the Flag DNA sequence plus 20 nucleotides complementary to the pcDNA3.1/zeo vector (Figure 3-2). The thermostable DNA polymerase used in this study was the *Pfu* DNA polymerase, not just because of its high fidelity (proof-reading activity) but also because it generates blunt-ended PCR products eliminating the need for end polishing. The PCR reaction was set up as follows:

10 X reaction buffer	5 µl
dNTPs (10 mM)	1 µl
Forward primer (100 pmol/µl)	1 µl
Reverse primer (100 pmol/µl)	1 µl
dd H_2O	36 µl
pcDNA 3.1/zeo rCLIC4 (2 ng/µl)	5 µl
Pfu Turbo DNA polymerase (2.5 U/ µl)	1 µl

Reverse primer (F - V):

5' P– **ATCCTTATAATC**CTTGGTAAGTCTCTTGGCGAC – 3'
½ Flag sequence

Forward primer (H1 - F):

5' P – **GACGACGACAAG**TAGCTCGACTCTAGAGGGC – 3'
½ Flag sequence

Flag sequence:

5' - GATTACAAGGAC **GACGACGACAAG** – 3'
D Y K D D D D K

Figure 3-2. The sequences of H1-F and F-V primers, and Flag epitope.

The bold nucleotides represent the first half (H1-F primer) and the second half (F-V) of the Flag sequence (24 nucleotides). The *Xho*I site is underlined.

Following an initial 94 °C denaturation step for 2 min, the reaction was cycled as follows: 94 °C, 30 sec; 60 °C, 30 sec; 72 °C, 2 min for 16 cycles. The reaction was terminated by a final extension step at 72 °C for 25 min.

Five microlitres of the PCR product were electrophorised on a 1 % (w/v) agarose gel to check for sufficient amplification (Figure 3-3). The PCR product was then ligated and treated with *Dpn I*. The *Dpn I* endonuclease (target sequence: 5'-$G^{m6}ATC$ -3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA sequence thus selecting the mutation-containing synthesized DNA. DNA isolated from almost all *E.coli* strains is dam-methylated and therefore susceptible to *Dpn I* digestion (Nelson and McClelland, 1992). The mutated vector DNA was subsequently transformed into XL1-Blue supercompetent cells. The integrity of DNA plasmid and absence of PCR induced errors were confirmed by sequencing over the entire coding region.

3.1.3 INDIRECT IMMUNOFLUORESCENCE

The intracellular localisation of rCLIC4 was examined by indirect immunofluorescence. HEK-293 cells were transiently transfected with pcDNA3.1/zeo rCLIC4 (full length) or pcDNA3.1/zeo Flag tagged rCLIC4 and stained with anti-CLIC4 or anti-Flag antibodies respectively, as described in section 2.4.3. Transfections were carried out using Lipofectamine reagent (Gibco) according to the manufacturer's instructions. Expression of rCLIC4 and rCLIC4-Flag could be detected 24 h post transfection (Figure 3-4). Confocal microscopy analysis of transfected HEK-293 cells demonstrated localisation of the tagged and untagged

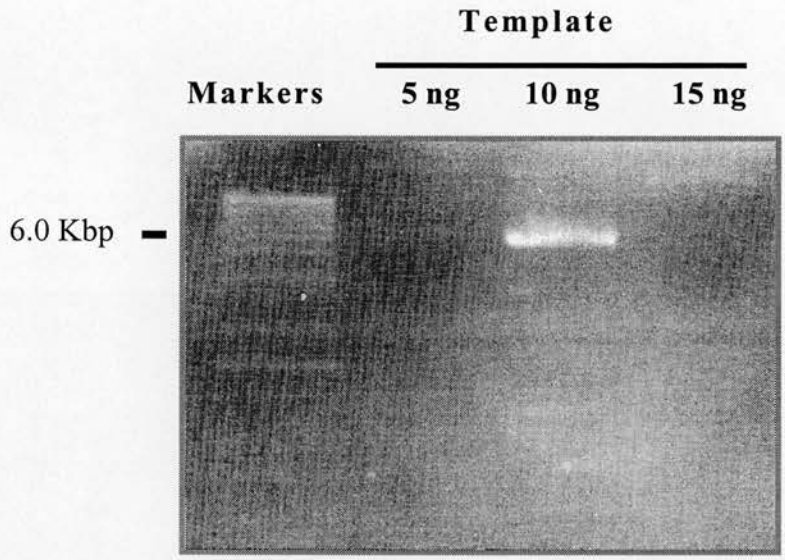


Figure 3-3. Generation of Flag-tagged rCLIC4 by Inverse PCR Mutagenesis.

The PCR reaction was repeated for 5, 10, 15 ng of pcDNA3.1/zeo rCLIC4 DNA template and run on 1 % (w/v) agarose gel as described in section 3.2.2.2. Successful amplification was detected using 10 ng template.

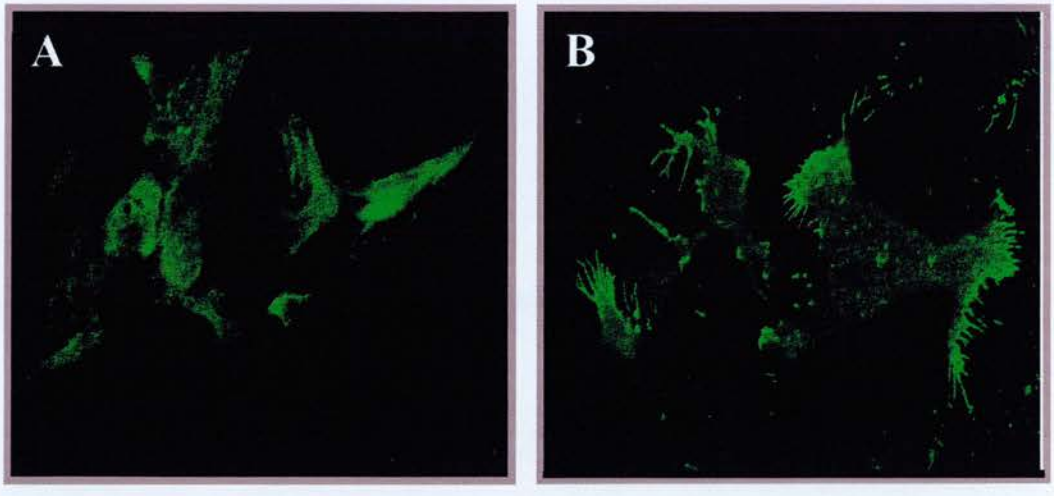


Figure 3-4. Indirect immunofluorescence detection of rCLIC4 in HEK-293 cells.

HEK-293 cells were transiently transfected using Lipofectamine reagent. Recombinant rCLIC4 (Panel A) and Flag tagged rCLIC4 (Panel B) were detected using rabbit polyclonal anti-CLIC4 and mouse monoclonal anti-Flag M2 primary antibodies respectively, stained with FITC-coupled secondary antibody. Fluorescent images were obtained using an upright Leica TCSNT confocal microscope (Leica Microsystems, Milton Keynes, UK) with a ×63 oil-immersion objective.

protein to the cytoplasm and membranous fractions – intracellular organelles and sites in the plasma membrane. To investigate whether the Flag epitope affects the expression pattern of rCLIC4 in HEK-293 cells and to further characterise the anti-CLIC4 immune serum, HEK-293 cells were transiently transfected with pcDNA3.1/zeo rCLIC4-Flag and cells were dual-stained with anti-Flag (TRITC) and affinity purified anti-CLIC4 (FITC) antibodies. Images were scanned and deconvoluted, and a co-localisation map was constructed as described in Figure 3-5. Staining for both anti-Flag and purified anti-CLIC4 was colocalised, with some additional staining observed for purified anti-CLIC4, possibly due to the expression of endogenous human CLIC4 in HEK293 cells. Transfection of HEK-293 cells with pcDNA3.1/zeo vector alone showed no detection of rCLIC4 by indirect immunofluorescence and no specific fluorescence was detected when rabbit pre-immune serum was used as the primary antibody. To further confirm the intracellular targeting of rCLIC4, Madine Derby Canine kidney (MDCK) epithelial-like cells (Gaush *et al.*, 1966) were used. Transfection and indirect immunofluorescence were repeated in the same manner as for HEK-293 cells. The pattern of staining observed in these experiments revealed expression of CLIC4 in both membrane and cytoplasmic locations (Figure 3-9)..

3.1.4 PRODUCTION OF HEK-293 CELLS STABLY TRANSFECTED WITH FULL LENGTH AND FLAG-TAGGED rCLIC4

In order to achieve high expression levels of rCLIC4 in HEK-293 cells, stable cell lines were established. The lethal concentration of the selection antibiotic,

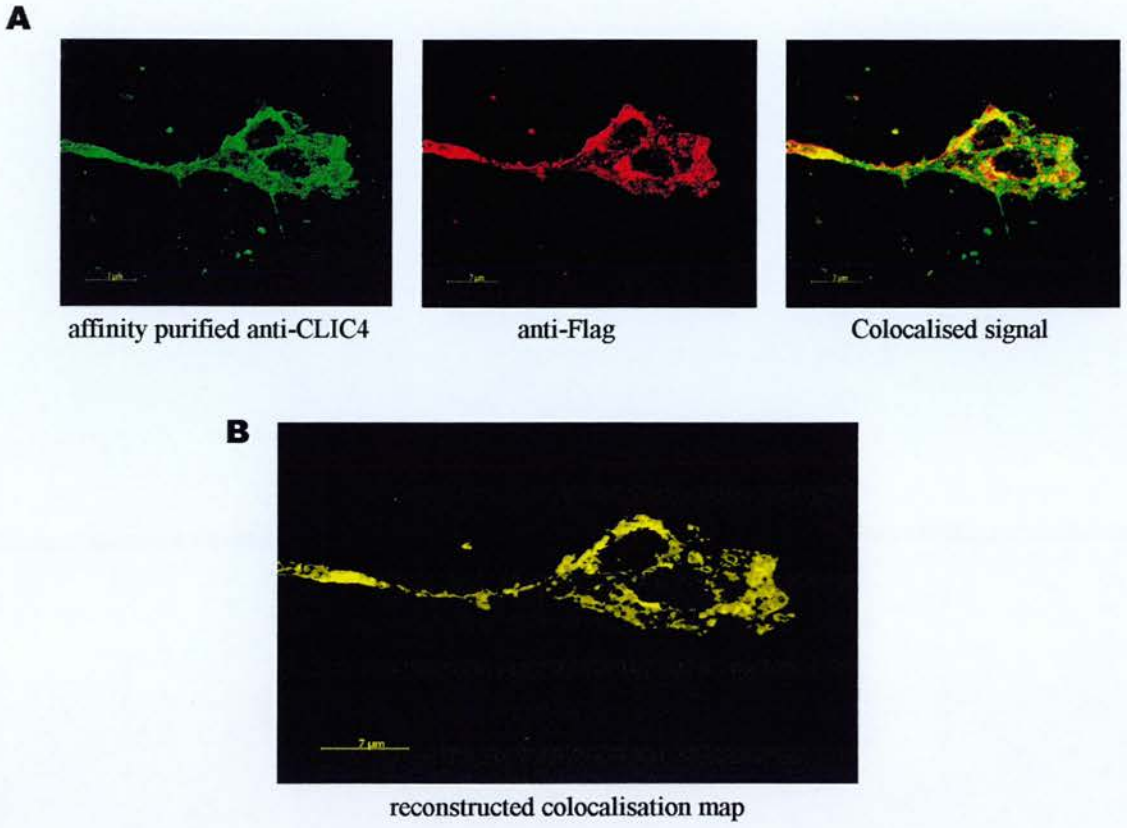


Figure 3-5. Indirect immunofluorescence detection of CLIC4 in HEK-293 cells by affinity purified anti-CLIC4 antibody.

HEK-293 cells were transiently transfected with pcDNA3.1/zeo CLIC4Flag as described before and dual-stained with affinity purified anti-CLIC4 (FITC) and anti-Flag (TRITC) antibodies (Panel A). Fixed cells on glass coverslips were imaged using simultaneous scanning with an excitation double dichroic beam splitter (488/568 nm FITC/TRITC). Image data acquired at Nyquist sampling rates were deconvoluted using the Huygens II package (Scientific Volume Imaging, The Netherlands) and analysed using the Bitplane suite of image analysis software (Bitplane AG, Zurich, Switzerland) on Silicon Graphics Octane 2 workstations. A quantitative, pixel-by-pixel analysis of co-localisation between data from each channel was performed using the Colocalisation package (v1.2, Bitplane AG). Pixels containing both green and red channel data were extracted from the entire 3-dimensional volume and reconstructed to give the colocalisation map shown in Panel B.

Zeocin (Invitrogen), was first determined for the non-transfected cells. HEK-293 cells were cultured in five wells of a six-well plate at similar density and grown for up to four weeks in DMEM / 10 % FBS media supplemented with 0, 0.1, 0.15, 0.2, 0.25 mg/ml Zeocin in each well respectively. The appropriate antibiotic concentration was 0.2 mg/ml culture media as determined by counting the number of dead cells (detached from the wells) in the four weeks culture period. At that concentration all cells were detached from the well within one week.

HEK-293 cells were then transfected with pcDNA3.1/zeo rCLIC4 (full length) or pcDNA3.1/zeo Flag tagged rCLIC4 using Lipofectamine reagent. Twenty-four hours after transfection, HEK-293 cells were passaged in a 12-well culture plate, in the presence of 0.2 mg/ml Zeocin in DMEM / 10 % FBS. The selection media was changed every five days and replaced with fresh media containing the same concentration of the antibiotic. Positive clones were selected approximately after four weeks. The selected cells were passaged into culture flasks and tested for rCLIC4 expression by immunoblotting (Figure 3-6). As shown in Figure 3.6 (Panel A) the affinity purified anti-CLIC4 antibody recognised a single band for the recombinant rCLIC4 in transfected HEK-293 cells and the endogenous human CLIC4 in control, non-transfected HEK293-cells.

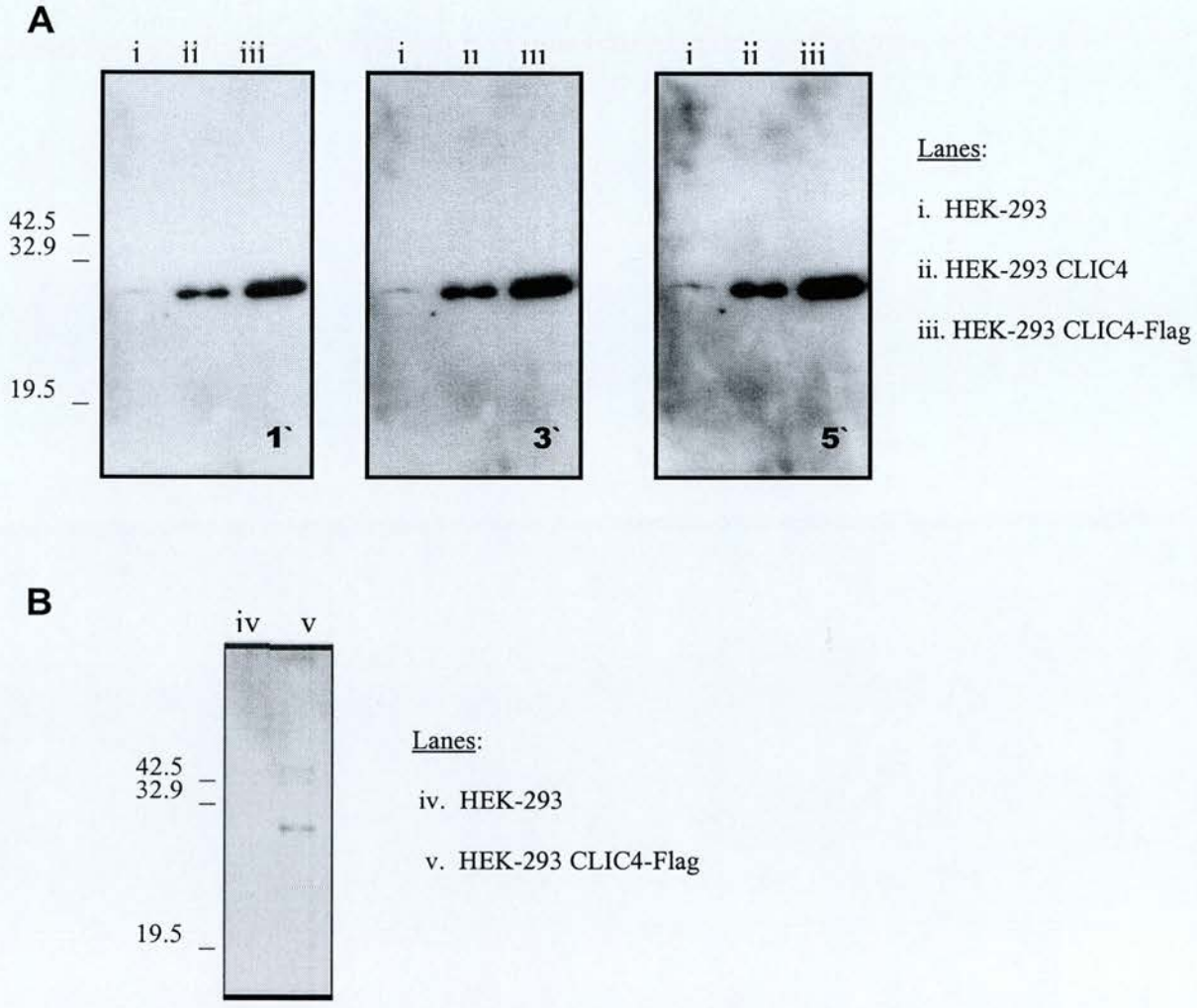


Figure 3-6. Expression of CLIC4 in stably transfected HEK-293 cells.

Positive clones from stably transfected HEK-293 cells with pcDNA3.1/zeo rCLIC4 (Panel A) and pcDNA3.1/zeo rCLIC4-Flag (Panel B) were isolated and the expressed proteins were detected with affinity purified anti-CLIC4 and monoclonal anti-Flag antibodies respectively. Equal volumes of cell lysates were loaded in each lane, and reactive proteins were revealed using an HRP-conjugated secondary antibody and ECL detection system (Amersham). To characterise the specificity of the affinity purified anti-CLIC4 antibody the membrane was exposed to the film for 1, 3 and 5 mins. The antibody recognised a single band at around 30 kDa, which corresponds to rCLIC4 and endogenous hCLIC4 (in HEK-293 cells).

3.1.5 SUBCELLULAR FRACTIONATION OF HEK-293 CELLS EXPRESSING rCLIC4

In order to determine the cellular location of rCLIC4 in non-transfected (control) HEK-293 cells and stably transfected with pcDNA3.1/zeo rCLIC4, cells were subjected to subcellular fractionation. HEK-293 cells were lysed in the presence of protease inhibitors (as described in section 2.3.7) and the pellet plus the supernatant fractions were collected by centrifugation at 1,000 *g*, 10,000 *g* and 300,000 *g* (at 4 °C) corresponding to the nuclei, mitochondrial and microsomal fractions respectively. Recombinant rCLIC4 was detected in these fractions by immunoblotting using the anti-CLIC4 polyclonal antiserum (Figure 3-7). Endogenous CLIC4 protein (human CLIC4) was also detectable by the same antibody (Figure 3-6), as human and rat CLIC4 have ~97 % amino acid homology (Chuang *et al.*, 1999). Both endogenous and recombinant CLIC4 appeared to have a dual localisation in membranous and cytosolic fractions, which is characteristic for other members of CLIC protein family.

3.2 rCLIC4 TARGETING TO CAVEOLAE DOMAINS

3.2.1 INTRODUCTION

Extensive studies of cell membrane structures have shown that microdomains exist within the fluid bilayer of the plasma membrane. These structures, known as lipid rafts, are rich in tightly packed sphingolipids and cholesterol (Simons and Ikonen, 1997). Different types of lipids rafts have been identified based on their.

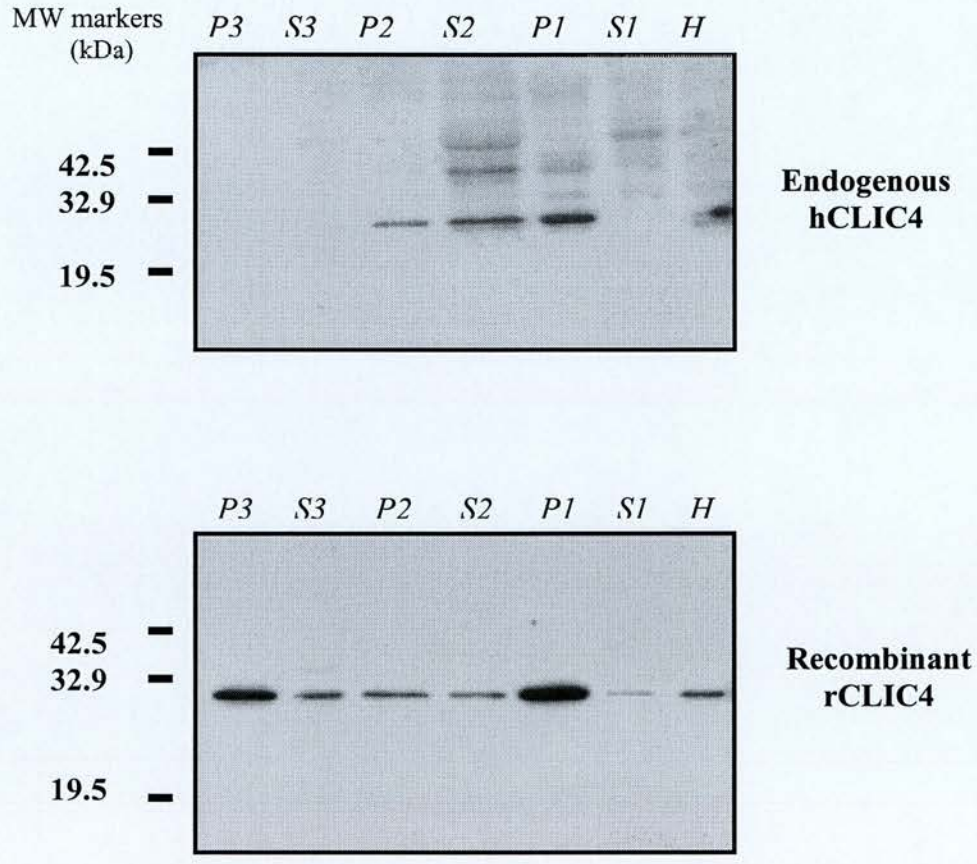


Figure 3-7. Subcellular distribution of rCLIC4 in HEK-293 cells.

Stably transfected and non-transfected HEK-293 cells were lysed in the presence of protease inhibitors and subjected to subcellular fractionation by centrifugation at 1,000 *g*, 10,000 *g* and 300,000 *g*. (H: homogenate; P: pellet; S: supernatant). Equal volumes from each fraction were analysed on a 12.5 % (w/v) SDS PAGE, proteins were transferred to PVDF membranes and CLIC4 protein was detected with anti-rCLIC4 antibody. Dual localisation in both cytosolic and membranous fractions were apparent for both endogenous (top) and recombinant (bottom) CLIC4.

structural characteristics and the presence of specific markers (reviewed by Brown and London, 1998). One well characterised type of lipid rafts is the caveolae. A number of definitions have been allocated to the term caveolae, which was first used by Yamada (1955) and defined as "a small pocket, vesicle, cave or recess communicating with the outside of the cell". Other definitions include both morphological and biochemical properties such as "flask shaped" or "omega shaped" and signalling domains (Lisanti *et al.*, 1995)Caveolae are known briefly as the 50 to 100 nm vesicular invaginations of the plasma membrane. The principal protein component of caveolae is caveolin, a 21 – 24 kDa membrane protein. Four different isoforms encoded by three genes have been identified: caveolin -1α, -1β, -2 and -3 (Parton, 1996). With the discovery of caveolin, as the first marker of caveolae (Rothberg *et al.*, 1992), new biochemical criteria were established for the identification and purification of these dynamic structures as described by (Shaul and Anderson, 1998). These include: a light buoyant density, Triton X-100 insolubility at 4 ^{o}C and enrichment in glycosphingolipids, cholesterol, sphingomyelin and lipid anchored membrane proteins. The effective isolation and purification of caveolae led to their functional characterisation. A number of signal transduction molecules have been described to localised in caveolae structures such as G protein mediated signalling molecules, calcium mediated signalling molecules, components of mitogen activated protein kinase pathways and lipid signalling molecules.

In this study the idea that rCLIC4 is targeting to caveolae fractions was tested by indirect immunofluorescence and immunoblotting of sucrose-density gradient isolated caveolae.

3.2.2 PARTIAL CO-LOCALISATION OF rCLIC4 AND CAVEOLIN

HEK-293 and MDCK were transiently transfected with pcDNA3.1 /zeo rCLIC4 and pcDNA3.1 /zeo rCLIC4-Flag respectively using Lipofectamine reagent. Co-localisation of expressed rCLIC4 with endogenous caveolin-1 was tested by indirect immunofluorescence as described before. MDCK cells were chosen as well as HEK-293 cells because they express a high level of caveolin-1. The fluorescence staining pattern of anti-caveolin antibody was similar in both cell types. The caveolin, marker of caveolae appeared in numerous punctuated spots that were concentrated along the edge and over the body of the cells. Confocal microscopy analysis of HEK-293 cells revealed a partial co-localisation of rCLIC4 and endogenous caveolin (Figure 3-8), whereas no co-localisation was detected in MDCK cells (Figure 3-9).

3.2.3 CAVEOLAE ISOLATION BY SUCROSE DENSITY CENTRIFUGATION

To further verify that rCLIC4 was present in caveolae membranes, a detergent based purification protocol was followed as described by Pike and Casey (1996). This method takes the advantage of the insolubility of caveolae in the presence of Triton X-100 at 4 ^{o}C. HEK-293 cells stably transfected with rCLIC4 were subjected to extraction in Triton X-100, followed by analysis of the lysate by sucrose density gradient centrifugation. One ml fractions were collected; equal volumes from each fraction were analysed on 12.5 % (w/v) SDS PAGE and immunoblotted with anti-caveolin-1 and anti-CLIC4 immune serum.

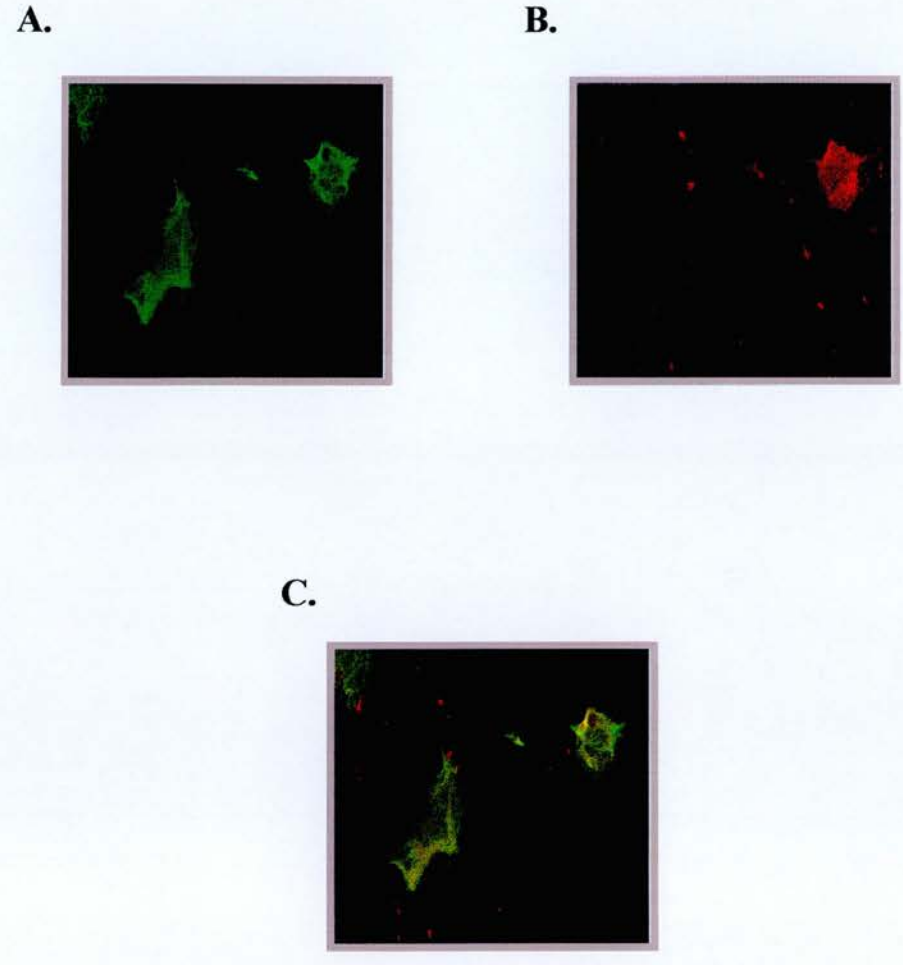


Figure 3-8. CLIC4 and caveolar endocytosis in HEK-293 cells.

Recombinant FITC- stained CLIC4 (**A**) and endogenous TRITC-stained caveolin-1 (**B**) are partial (yellow) co-localised (**C**) in transiently transfected HEK-293 cells.

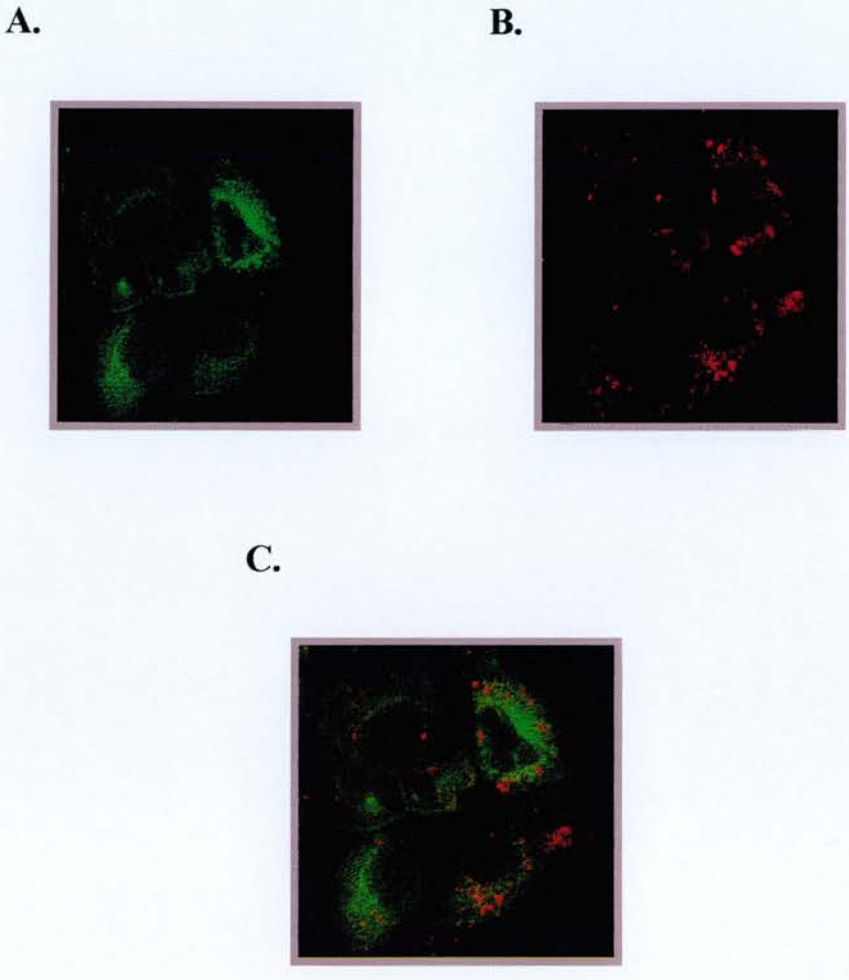


Figure 3-9. CLIC4 and caveolar endocytosis in MDCK cells.

Recombinant FITC- stained CLIC4 (**A**) and endogenous TRITC-stained caveolin-1 (**B**) show distinct localisation (**C**) in transiently transfected MDCK cells.

Figure 3-10 shows a characteristic distribution caveolin, the marker of caveolae. The vast majority of caveolin appeared to be in fractions 4-6, which correspond to the interface between 5 % and 30 % (w/v) sucrose layers. A lower amount of caveolin was detected in fractions 9-11, probably due to the presence of free caveolin (present before the biogenesis of caveolae; Lisanti *et al.*, 1995) or due to the association of caveolin with cytoskeletal elements (Pike and Casey, 1996). As shown in Figure 3-9, the distribution of rCLIC4 was distinct from that observed for caveolin. However immunoblotting analysis of concentrated fractions 4-6 by precipitation with 30 % (w/v) trichloroacetic acid (TCA) revealed that rCLIC4 was detected in low levels. This result is consistent with the finding that rCLIC4 and caveolae are partial co-localised in HEK-293 cells.

3.3 SUMMARY

Rat CLIC4 cDNA (Duncan *et al.*, 1997) was subcloned into pcDNA3.1/zeo mammalian expression vector and a Flag tag was fused at the carboxyl terminus by inverse PCR mutagenesis. HEK-293 cell lines stably transfected with Flag-tagged and non-tagged rCLIC4 were established, expressing high level of rCLIC4 protein as shown by immunoblotting. Indirect immunofluorescence in HEK-293 and MDCK cells and subcellular fractionation studies, showed dual localisation of heterologously expressed rCLIC4 in membranous and cytoplasmic fractions. The additional immunoreactive signal identified by the affinity purified anti-CLIC4 antibody beyond that detected by the Flag antibody is shown clearly by the green (FITC) signal still present in the colocalised image (Figure 3-5 Panel A).

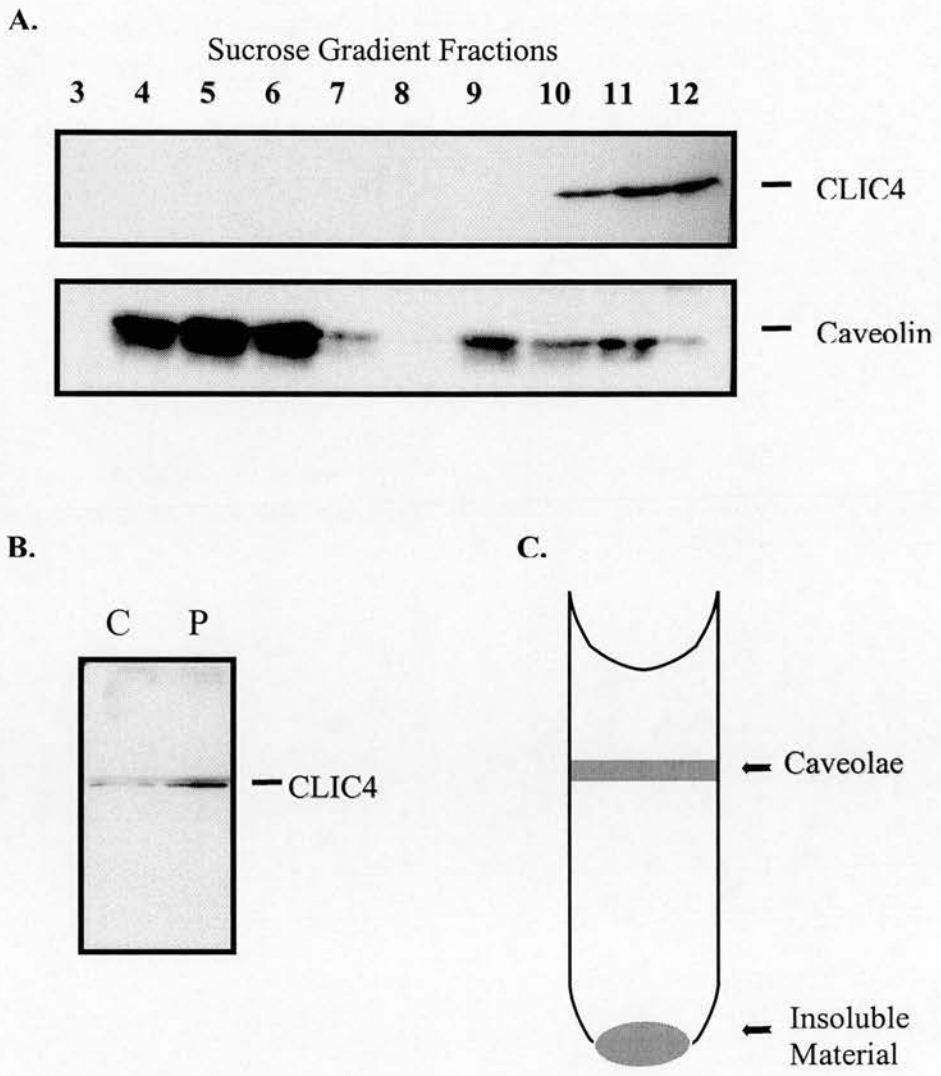


Figure 3-10. Detection of recombinant CLIC 4 in caveolae membranes.

Panel A. HEK-293 lysate was subjected in sucrose density gradient centrifugation and the resulting fractions were tested by immunoblotting with anti-CLIC4 (top) and anti-caveolin-1 (bottom) immune serum; **Panel B.** Caveolae fractions 4-6 (C), concentrated by TCA precipitation and pellet (P) blotted with anti-CLIC4; **Panel C.** Diagrammatic representation of caveolae position in the sucrose density gradient.

The evidence presented in Figure 3-6, that the affinity purified anti-CLIC4 antibody recognises only a single band indicates that the additional immunoreactivity is unlikely to represent a non-specific interaction and could represent the low levels of endogenous CLIC4. When immunofluorescence was performed with crude anti-CLIC4 immune serum (Figure 3.4) there were also differences in the subcellular staining pattern compared to that seen with the Flag antibody. In that case, the ability of the antibody to cross react, to a minor extent, with additional proteins (Figure 3.7) raises the possibility that some of the signal may be non-specific. However the fact that similar results were obtained with the affinity purified antiserum, suggests that there is an authentic pool of CLIC4 in cells additional to the Flag staining observed following transfection of the tagged construct. Finally, partial co-localisation of rCLIC4 and endogenous caveolin, a marker of caveolae domains, was demonstrated by indirect immunofluorescence in HEK-293 cells but not in MDCK cells and verified by immunoblotting of sucrose density gradient isolated caveolae fractions.

CHAPTER 4

IDENTIFICATION OF PROTEINS ASSOCIATED WITH rCLIC4

4.1 PROTEIN AMPLIFICATION AND PURIFICATION

4.1.1 INTRODUCTION

The need for stable and soluble recombinant proteins and their subsequent purification, under non-denaturing conditions, has led to the construction of several vectors that simplify the expression, purification and detection of fusion proteins produced by *E. coli*. The two most commonly used tags are glutathione-S-transferase (GST) and 6x histidine $(HIS)_6$. In this study we used the GST gene expression system, which combines all the advantages described above. Full length and truncated (cytoplasmic domain) rCLIC4 cDNAs were cloned into the pGEX 4T.1 and pGEX 6P.1 vectors (Pharmacia). The expression of fusion proteins is under the control of the *lac* promoter, which is induced using the lactose analogue isopropyl β-D-thiogalactoside (IPTG). Fusion proteins are easily then purified from bacteria lysates by affinity chromatography using glutathione Sepharose 4B (GS4B) Fast Flow (Pharmacia).

GST occurs naturally as a 26 kDa protein encoded by the parasitic helminth *S. japonicum*, and can be expressed in *E. coli* with full enzymatic activity (Smith and Johnson, 1988). The GST tag can be used in any expression system and gives a high yield of pure products that can be purified using a simple protocol (Figure 4-1). Very mild elution conditions are required, minimizing the risk of functionally damaging the target protein. The GST tag can help stabilize folding of the recombinant proteins and can easily be detected using an enzyme assay or an immunoassay. Dimer formation is usual with GST fusion proteins since GST itself is homodimer when folded (Smith and Johnson, 1988).

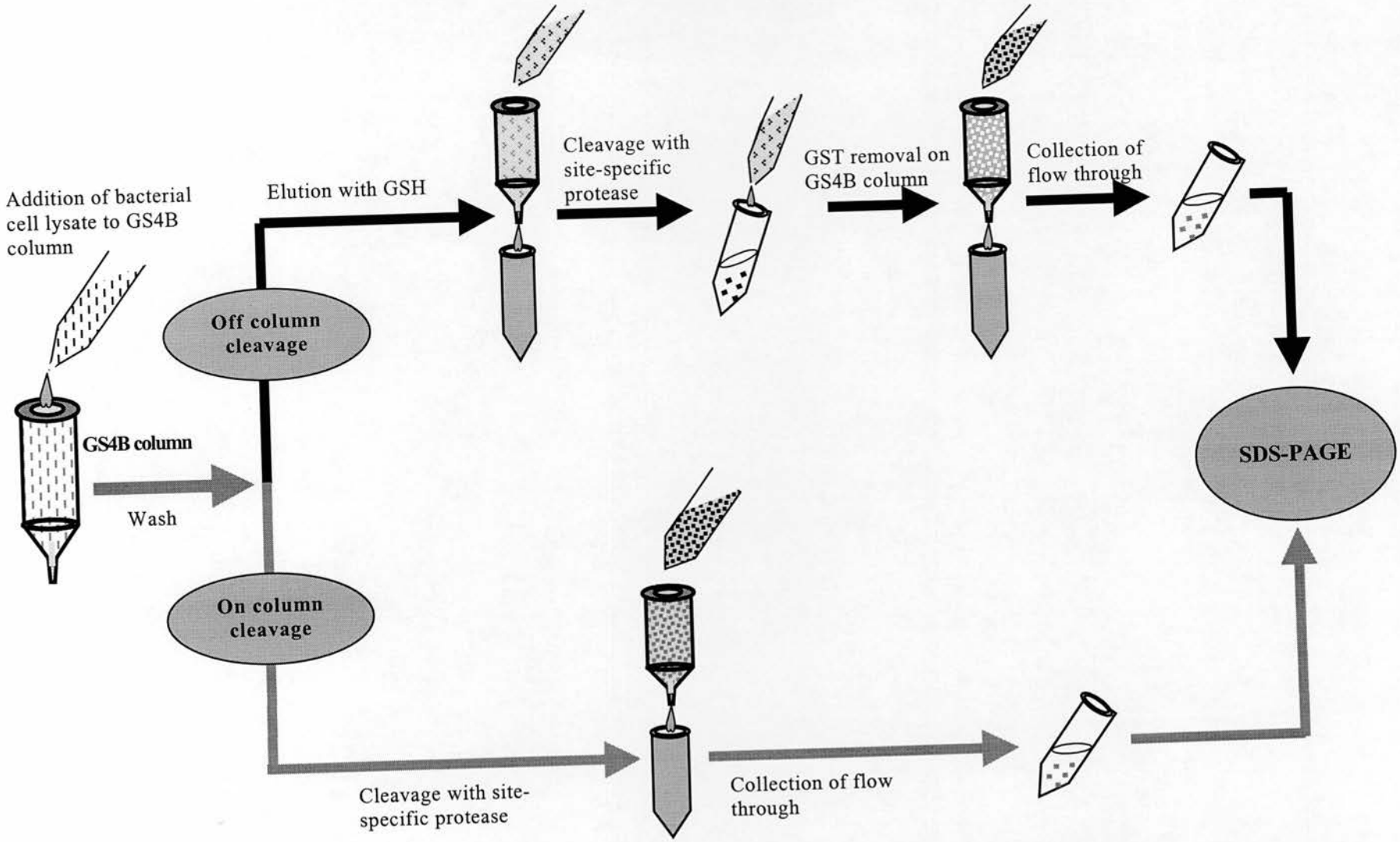


Figure 4-1. Flow chart of the affinity purification procedure and Thrombin or PreScission Protease cleavage of GST-CLIC4.

Cleavage of GST tag, when required, is achieved using a site-specific protease such as thrombin, Factor Xa, and Prescission proteases, depending on the expression vector used (pGEX-4T, pGEX-5X and pGEX-6P respectively), each with a unique cleavage recognition site (see Appendix I for maps of vectors). pGEX-6P PreScission protease vectors offer the most efficient method for cleavage and purification of GST fusion proteins. PreScission protease is a fusion protein of GST and human rhinovirus 3C protease. The advantage of PreScission protease over thrombin and Factor Xa is that since the protease is fused to GST, it is easily removed from cleavage reaction. Furthermore it has the ability to retain its high activity at low temperatures so that all steps can be performed at 4 ^{o}C to maintain protein integrity.

4.1.2 EXPRESSION AND PURIFICATION OF FULL LENGTH AND TRUNCATED (CYTOPLASMIC DOMAIN) rCLIC4

A cDNA encoding the cytoplasmic domain (residues 60-254) of rCLIC4 cDNA (Howell *et al.*, 1996; Duncan *et al.*, 1997) was amplified from full-length rat brain p64H1 cDNA by PCR, using the oligonucleotides:

5'-CGCGGATCCAAAAGGAAGCCTGCACATCTGCAGAACTTGGC-3' and

5'-ATACCGCTCGAGCTACTTGGTAAGTCTCTTGGCGACG-3'.

The insert was cloned into pGEX-4T1 and pGEX-6P1 expression vectors using engineered 5' *Bam*H I and 3' *Xho* I sites (underlined), to create an in-frame fusion with the sequence encoding GST. The recombinant plasmid was transformed into *E. coli* DH5α cells and the clones were isolated. Positive results for the integrity of the cDNA insert and the correct in-frame ligation were confirmed by sequencing. A single colony was inoculated into 100 ml LB media containing 100 μg/ml ampicillin and grown at 37 °C until the OD_{600} reached 0.6. Expression of the protein was induced by addition of IPTG (final concentration 1 mM) at 25 °C. Bacterial cells were lysed by sonication in the presence of protease inhibitors and the supernatant plus the pellet fractions were collected by centrifugation (30 min at 6,000 *g*, 4 °C) as described in section 2.2.3. In each case, synthesis of an abundant GST fusion protein of the predicted size (54 kDa for the GST-rCLIC4 full length, 48 kDa for the GST-rCLIC4 cytoplasmic domain and 26 kDa for GST alone) was observed by SDS PAGE (followed by Coomassie Brilliant Blue staining) two and three hours after induction (Figure 4-2, Panel A). Analysis of supernatant and pellet fractions of lysed bacteria revealed as expected that the recombinant protein was almost completely soluble.

GST fusion proteins were purified from crude bacterial lysates under non-denaturing conditions by affinity chromatography on immobilised glutathione, as described in section 2.3.6. Purified fusion proteins were eluted from the GS4B Fast Flow column with reduced glutathione buffer and the samples were analysed by SDS PAGE followed by Coomassie Brilliant Blue staining (Figure 4-2, Panel B).

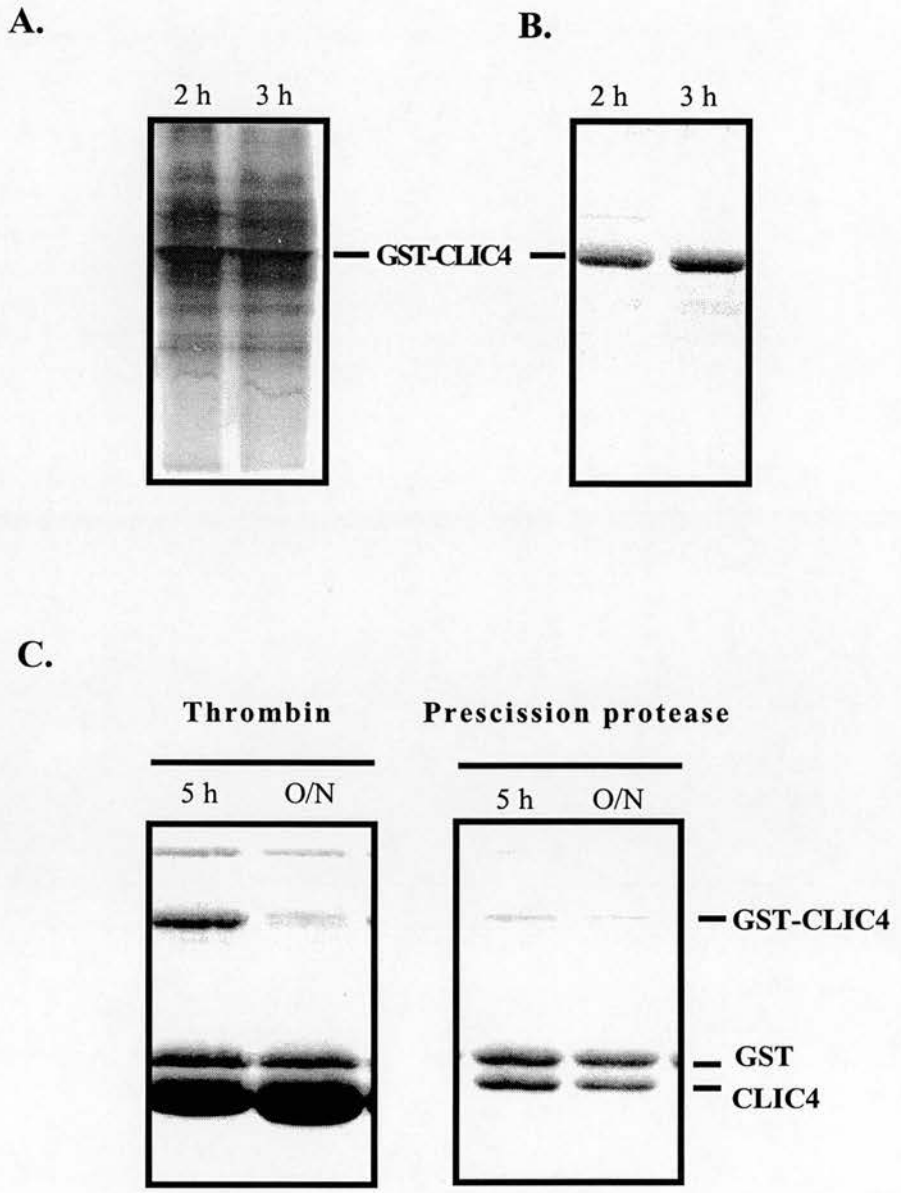


Figure 4-2 Expression, purification and enzymatic cleavage of GST-CLIC4.

Panel A. Lysates from IPTG–induced bacteria (2 and 3 hours after induction) were run on 10 % (w/v) SDS-PAGE followed by staining with Coomassie Brilliant Blue.

Panel B. SDS-PAGE analysis of GST-CLIC4 fusion protein bound on GS4B beads eluted with reduced glutathione buffer (2 and 3 hours after induction with IPTG).

Panel C. SDS-PAGE analysis of the on-column GST-tag enzymatic cleavage after 5 hours and overnight treatment with Thrombin and Prescission protease at 4 ^{o}C.

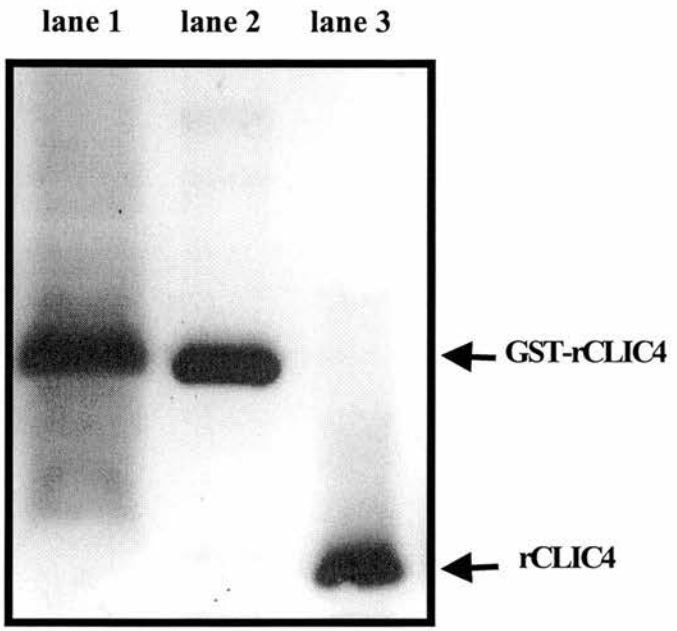


Figure 4-3. Western blot of GST tagged and untagged rCLIC4.

Lane 1: Bacterial extract, Lane 2: purified GST-CLIC4 on GS4B column Lane 3: cleaved CLIC4. Five micrograms of proteins were loaded per well, analysed on 10 % (w/v) SDS-PAGE and immunoblotted with rabbit anti-CLIC4 polyclonal antibody.

4.1.3 PROTEASE CLEAVAGE OF PURIFIED FUSION PROTEINS

In most cases, functional tests were performed using the intact GST fusion proteins. When cleaved recombinant proteins were required, removal of the GST tag was achieved by enzymatic cleavage (Figure 4-2, Panel C and Figure 4-3). The enzymes used were thrombin and PreScission protease, depending on the pGEX expression vector (pGEX-4T1 and pGEX-6P1 respectively) and the optimal cleavage conditions were identified in each case as shown below in Table 4-1.

Table 4-1 Optimal conditions for GST-tag enzymatic cleavage by Thrombin and PreScission Protease.

Cleavage conditions	Thrombin	PreScission Protease
CLEAVAGE BUFFER	PBS pH 7.4	150 mM NaCl 50 mM Tris-HCl 1 mM EDTA 1 mM DTT pH 7.0
INCUBATION	2 hours at RT O/N at 4 °C	5 hours at 4 °C
REMOVAL OF ENZYME	Biotin trap	Not necessary

4.2 ISOLATION OF rCLIC4 ASSOCIATED PROTEINS FROM RAT BRAIN CYTOSOL

In order to isolate proteins associated with rat brain CLIC4, GST-rCLIC4 fusion proteins (full length and cytoplasmic domain) immobilised on GS4B column were incubated with rat brain cytosol. These experiments were carried out in collaboration with W. Suginta. The rCLIC4-protein complex samples were analysed on 4-12 % (w/v) gradient SDS-PAGE, followed by in-gel digestion with trypsin (Promega) for two hours. The resulting peptides were concentrated in a SpeedVac vacuum centrifuge, desalted by microbore reverse phase HPLC (C_{18}) and analysed on a Thermoquest LCQ ion-trap mass spectrometer in nanospray mode. MS/MS analysis of the digested protein fragments with Sequest software, revealed a number of proteins (Figure 4-4) that were able to bind to rCLIC4 directly or indirectly. These included dynamin I, α-tubulin, β-actin, creatine kinase β-chain and the two 14-3-3 isoforms (ε and ζ).

4.3 REVERSE PULL DOWN ASSAYS

To confirm the direct interaction between rCLIC4 and 14-3-3ζ, reverse pull down assays were carried out. rCLIC4 cytoplasmic domain was incubated overnight at 4 °C with immobilized GST-(14-3-3ζ) to glutathione beads in the presence of binding buffer as described in Materials and Methods. Following extensive washing of the beads with binding buffer containing 2M NaCl, the GST-(14-3-3ζ) / rCLIC4 protein complex was eluted by addition of 2x SDS loading buffer. The samples were subsequently subjected to SDS-PAGE and immunoblotting using anti-CLIC4 immune serum, as shown in Figure 4-5 (Panel A, lane 1).

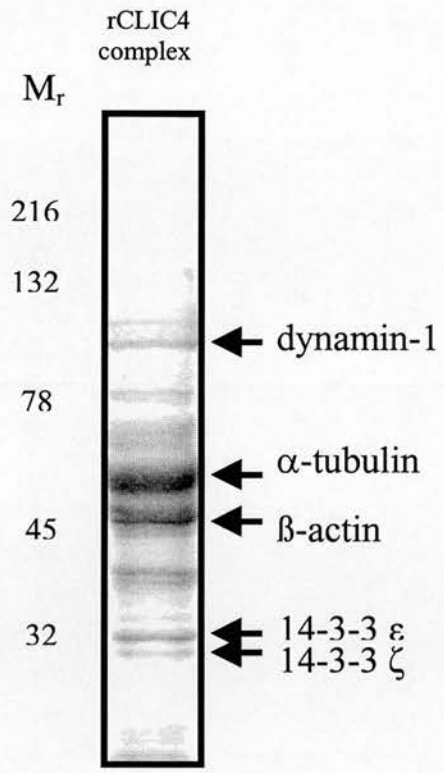


Figure 4-4. Identification rCLIC4 associated proteins.

The rat brain cytosol was incubated with rCLIC4 cytoplasmic domain fused to GST and attached to glutathione beads in the presence of protease inhibitors. After extensive washing the samples were loaded on SDS-PAGE, followed by in-gel trypsin digestion and microbore reverse-phase HPLC. Subsequent MS/MS analysis of the digested segments revealed six proteins in the rCLIC4 complex. These included dynamin I, α-tubulin, β-actin and the ε and ζ isoforms of 14-3-3. (This experiment was carried out by Dr W Suginta).

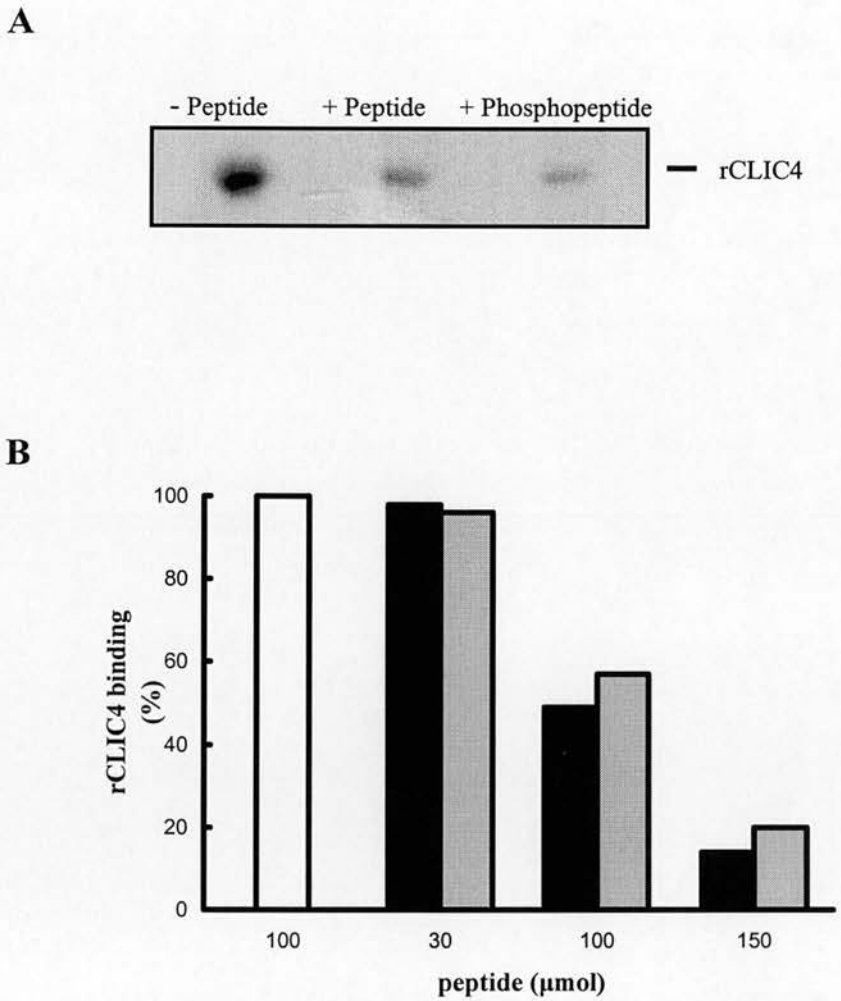


Figure 4-5. Competition binding assay with immobilized 14-3-3ζ.

Panel A. rCLIC4 binding to immobilised 14-3-3ζ in the absence of competing peptides (- Peptide). In the presence of 150 μmol Raf peptide (+ Peptide, + Phosphopeptide), binding of rCLIC4 to 14-3-3ζ is decreased.

Panel B. Graphical representation of percentage (%) rCLIC4 binding to 14-3-3ζ in the presence of 0, 30, 100 and 150 μmol Raf phosphorylated peptides (black bars) and non-phosphorylated peptides (grey bar). rCLIC4 binding was not decreased by 100 μmol of control peptide (white bar). Each bar is the mean value from two independent experiments.

To further characterise the direct interaction of rCLIC4 with 14-3-3ζ, the binding assay was repeated in the presence of a 10-fold molar excess of one of two synthetic peptides corresponding to the Raf 14-3-3 binding sequence LSGRGRSTSTPNVHMV, where the underlined serine residue was either phosphorylated or non-phosphorylated. As shown in Figure 4-5 (Panel A), in the presence of 150 μmol of the non-phosphorylated competing Raf peptide, rCLIC4 binding was reduced. This was more marked when using the phosphopeptide. The competition assays were repeated in the presence of 30 μmol and 100 μmol of Raf peptides (phosphorylated and non-phosphorylated), and 100 μmol of a control peptide NVVGARRSSWRVISSIEQKT (residues 51-70 of the γ isoform of 14-3-3). The signals were densitometrically (Advance Image Data Analyzer – AIDA 2.0 software) compared with the non-inhibited binding (Figure 4-5, Panel B).

4.4 IMMUNOPRECIPITATION OF rCLIC4-ASSOCIATED PROTEINS

As described in section 1.5.7, Northern and Western blot analysis of rCLIC4 revealed that the protein is highly expressed in rat brain, particularly in the cerebellum (Duncan *et al.*, 1997). To confirm that the identified proteins bind to rCLIC4 *in vivo* as well as *in vitro* (either directly or indirectly), immunoprecipitation studies were performed with rat brain cytosol and transfected HEK-293 cells which express high levels of rCLIC4 or FLAG tagged rCLIC4.

The presence of detergents (0.5-1 % (v/v) Triton X-100, 1 % (w/v) CHAPS or 1 % (w/v) n-octyglucoside) in the immunoprecipitation buffer decreased the binding of rCLIC4 to its protein partners. As a result, the association of rCLIC4 with membrane-bound proteins could not be demonstrated using the immunoprecipitation technique.

4.4.1 rCLIC4 BINDING PARTNERS IN RAT BRAIN CYTOSOL

Rat brain cytosol was prepared as described previously (Duncan *et al.*, 1997) in the presence or absence of detergents (0.5-1 % (v/v) Triton X-100, 1 % (w/v) CHAPS or 1 % (w/v) n-octyglucoside in lysis buffer). One ml of rat brain cytosol containing protease inhibitors was pre-cleared by the addition of Protein G-Sepharose beads (50 % (v/v) slurry in PBS) and incubated at 4 ^{o}C for 30 min followed by brief centrifugation to remove the slurry. The pre-cleared rat brain cytosol was mixed with the specified dilution of anti-CLIC4 antiserum in PBS and the immunoprecipitation was proceeded as described previously.

Immunoprecipitated β-actin, α-tubulin and 14-3-3ζ proteins were detected using the appropriate non-rabbit antibodies (Table 4-2, Figure 4-6). As a commercial non-rabbit 14-3-3ε antiserum was not available to detect the 14-3-3ε isoform from the protein-antibody complex, a recently raised sheep anti-(14-3-3ε) antiserum was used (a kind gift by Dr S. Mackie) but failed to detect the protein from the rat brain cytosol (control) and the immunocomplex.

Proteins	Immunoprecipitated antibody	Detection antibody	Binding to rCLIC4
Dynamin 1 [(a)]	Rabbit anti-CLIC4	Sheep anti- Dyn 1	+
α–Tubulin	Rabbit anti-CLIC4	Mouse anti- (α) tubulin	+
β–Actin	Rabbit anti-CLIC4	Mouse anti- (β) actin	+
14-3-3ζ	Rabbit anti-CLIC4	Sheep anti- (14-3-3ζ)	+
14-3-3ε	Rabbit anti-CLIC4	Sheep anti- (14-3-3ε)	*

[(a)] Carried out by Dr W. Suginta

+, protein detected in the immunocomplex;

Table 4.2 **Summary of the proteins tested and the antibodies used in the immunoprecipitation studies from rat brain cytosol.**

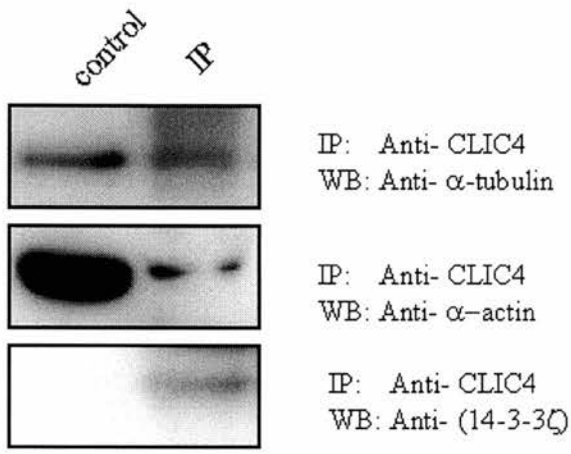


Figure 4-6. Immunoprecipitation of CLIC4 binding proteins from rat brain.
α-tubulin, α–actin and 14-3-3ζ were immunoprecipitated (IP) from rat brain cytosol by rabbit polyclonal anti-CLIC4 antiserum as shown in the figure. The immunocomplexes were captured on protein G beads and subjected to SDS-PAGE and immunoblotting (WB) as described in Materials and Methods.

4.4.2 rCLIC4 BINDING PARTNERS IN MAMMALIAN CELLS

HEK-293 cells stably expressing high levels of rCLIC4 or Flag-tagged rCLIC4 were grown to subconfluence (70-80 %), followed by lysis in the presence of protease inhibitors. Immunoprecipitation was performed as described in Materials and Methods, using either rabbit anti-CLIC4 polyclonal or mouse anti-Flag M2 monoclonal antiserum diluted to the appropriate concentration (usually 1:100 in lysis buffer).

As dynamin I is mainly expressed in neurones (Torre *et al.*, 1994) and because HEK-293 is an epithelial cell line, it is unlike that the protein-antibody complex could contain dynamin I. When immunoprecipitations were carried out with the rabbit anti-CLIC4 or mouse anti-FLAG antiserum, dynamin I could not be detected by the sheep anti-dynI antiserum in western blot analyses. However, HEK-293 cells express dynamin II (Cook *et al.*, 1994). To test whether rCLIC4 associates with dynamin II, immunoprecipitations were repeated as before but this time a sheep anti-dynII antiserum (a kind gift from Dr PJ Robinson, Children's Medical Research Institute, Australia), that was raised against a recombinant dynamin II peptide, was used for detection in western blots. The anti-dynII antiserum failed to detect dynamin II both in HEK-293 lysate (control) and in the immunocomplex. The immunoprecipitation results and the antibodies used in each case are summarised in Table 4-3. Results from the immunoprecipitated proteins rCLIC4 and the 14-3-3ζ isoform are shown in Figure 4-7.

Proteins	Immunoprecipitating antibody	Detection antibody	Binding to rCLIC4
rCLIC4	Rabbit anti-CLIC4	Mouse anti- Flag	+
	Mouse anti-Flag	Rabbit anti-CLIC4	+
Dynamin I	Rabbit anti-CLIC4	Goat anti- DynI	*
	Mouse anti- Flag	Rabbit anti- DynI	*
Dynamin II	Rabbit anti-CLIC4	Sheep anti- DynII	*
	Mouse anti- Flag		*
α–Tubulin	Mouse anti- Flag	Rabbit anti- (α) tubulin	+
β–Actin	Rabbit anti-CLIC4	Mouse anti- (β) actin	-
14-3-3ζ	Mouse anti- Flag	Sheep anti- (14-3-3)	+
Caveolin 1	Rabbit anti-CLIC4	Mouse anti-caveolin 1	-
	Mouse anti-Flag	Rabbit anti-caveolin 1	-

+, protein detected in the immunocomplex;

-, protein could not be detected in the immunocomplex;

*, protein could not be detected neither from the control HEK-293 lysate or the immunocomplex

Table 4-3. Summary of the proteins tested and the antibodies used in the immunoprecipitation studies from HEK-293 lysate.

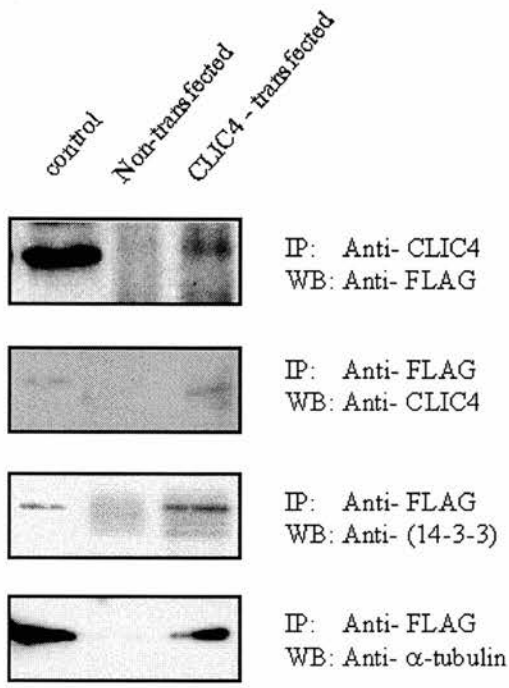


Figure 4-7. Immunoprecipitation of rCLIC4 binding proteins from HEK-293. rCLIC4 (Flag tagged and untagged), 14-3-3 and α-tubulin were immunoprecipitated (IP) from HEK-293 cells stably transfected with rCLIC4 by rabbit polyclonal anti-CLIC4 or mouse monoclonal anti-Flag antibodies. The immunocomplexes were captured on protein G beads and subjected to SDS-PAGE and immunoblotting (WB) as described in Materials and Methods. No signal was obtained in immunoprecipitation controls with non-transfected HEK-293 cells.

4.5 ACTIN CO-SEDIMENTATION ASSAY

To further examine the association of rCLIC4 with actin, actin co-sedimentation assays were performed with platelet β-actin and γ-actin, as described in Materials and Methods. Figure 4-8 (Panel B) shows that rCLIC4 cytoplasmic domain remained in the supernatant fraction and did not co-sediment with polymerised F-actin unlike cofilin (a kind gift from Dr S. Maciver, Edinburgh), which bound readily to (β/γ)-actin (Figure 4-8, Panel A). This suggests that rCLIC4 might interact indirectly with β-actin rather directly or that it might bind to monomeric G-actin. Similar results were obtained when the assay was repeated at low pH (pH 6.5) and with rabbit muscle α-actin.

4.6 *IN VITRO* AND *IN VIVO* PHOSPHORYLATION OF rCLIC4

As discussed previously, several consensus phosphorylation sites were predicted to reside within the cytoplasmic domain of rCLIC4, including PKC, PKA, casein kinase II and tyrosine kinase sites. To examine PKC-, PKA- and CK II-mediated phosphorylation *in vitro*, recombinant GST-rCLIC4 was affinity purified on GS4B column, cleaved from the GST tag and resuspended in phosphorylation buffer in the presence of radiolabelled [^{32}P] γ-ATP. The samples were electrophorised in a 12 % (w/v) SDS-PAGE and exposed to autoradiographic film (Figure 4-9).

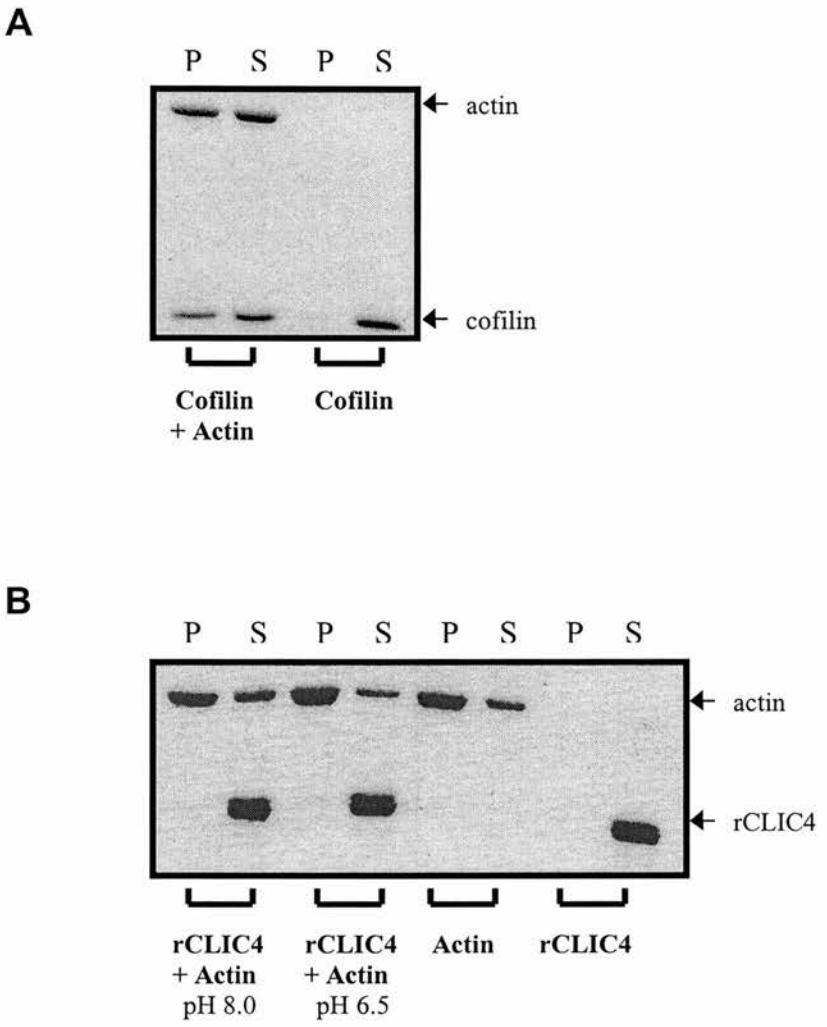


Figure 4-8. Actin co-sedimemntation assay.

G-actin was allowed to polymerise with or without cofilin (an actin binding protein) at pH 6.5 (Panel A) and with or without rCLIC4 (cytoplasmic domain) at pH 6.5 and pH 8.0 (Panel B). After centrifugation polymerised proteins, containing F-actin were pelleted (P), whereas non-polymerised proteins, containing G-actin remained in the supernatant fraction (S). Equal volumes of the pellet and the supernatant fractions were subjected to 10 % (w/v) SDS-PAGE and Coomassie staining. Unlike cofilin, rCLIC4 remains in the supernatant fraction and does not co-sediment with actin.

In addition to *in vitro* phosphorylation experiments, rCLIC4 was shown to be *in vivo* phosphorylated by tyrosine kinase. Transfected HEK-293 cells expressing rCLIC4 and non-transfected (control) cells were grown to subconfluence (70-80 %) and treated with tyrosine phosphatases inhibitors (section 2.5.5.3) prior to lysis. HEK-293 lysates from cells treated with tyrosine phosphatase inhibitors were analysed by SDS-PAGE. Proteins phosphorylated at tyrosine residues were detected by immunoblotting using a phosphotyrosine monoclonal antibody (Cell Signalling). To investigate whether rCLIC4 is phosphorylated by tyrosine kinases, anti-CLIC4 immune serum was added to the lysate as described previously, and the immunoprecipitated rCLIC4 was detected by immunoblotting using a phosphotyrosine monoclonal antibody (Figure 4-10).

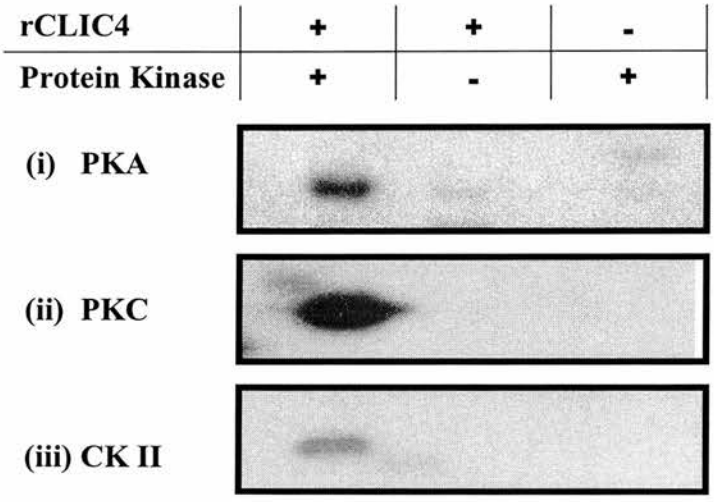


Figure 4-9. rCLIC4 *in vitro* phosphorylation assays.

Reaction mixtures were incubated with [γ-^{32}P] ATP in the presence (+) or absence (-) of purified recombinant rCLIC4, and in the presence or absence of protein kinases (protein kinase A, protein kinase C and casein kinase II) for 10 mins at 30 ^{o}C. The reactions were stopped by the addition of SDS-loading buffer and the samples were run on 12 % (w/v) SDS-PAGE gels. The gels were allowed to dry and then they were exposed to the [γ-^{32}P]-sensitive film (KODAK).

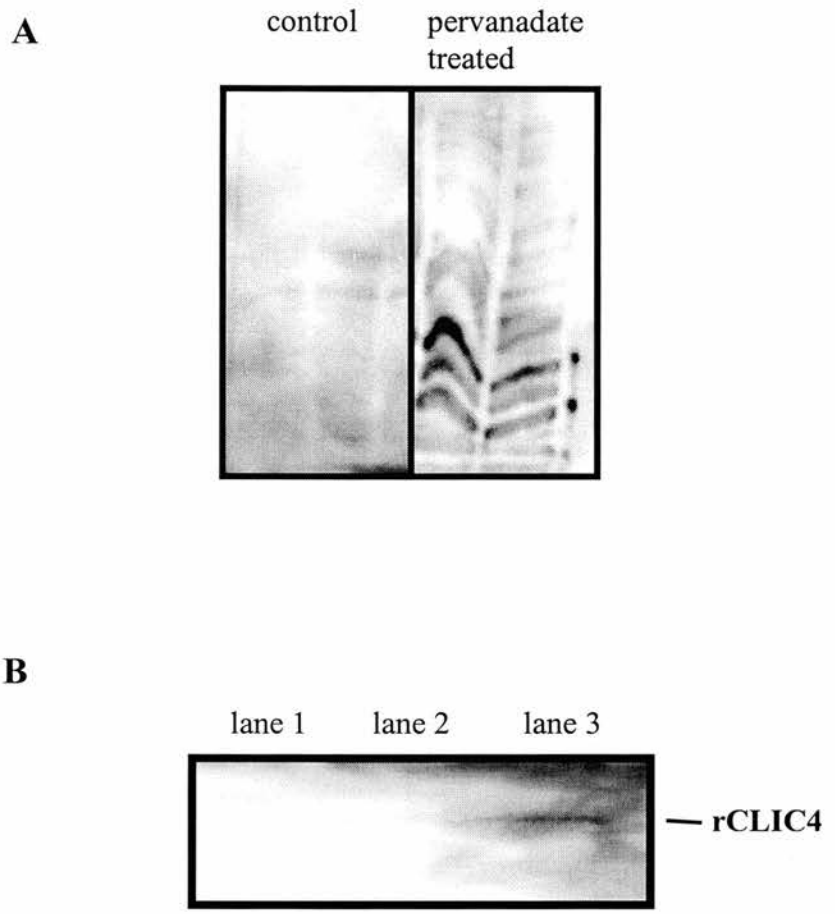


Figure 4-10. Tyrosine kinase-mediated phosphorylation of rCLIC4.

PANEL A. HEK-293 lysates, from pervanadate-treated cells, and control (non-treated cells) were analysed on a 10 % (w/v) SDS-PAGE gel. Proteins phosphorylated at tyrosine residues were detected by immunoblotting with phosphotyrosine (PY) monoclonal antibody.

PANEL B. rCLIC4 was immunoprecipitated from pervanadate-treated HEK-293 stably transfected cells, and detected by immunoblotting with phosphotyrosine (PY) monoclonal antibody (lane 3). No signals were detected from pervanadate-treated, non-transfected cells (lane 1) and from non-treated, stably transfected cells (lane 2).

4.7 SUMMARY

Full length and truncated (cytoplasmic domain) rCLIC4 were amplified by PCR and the inserts were cloned into pGEX vectors. Optimal conditions were determined for the expression and purification of GST fusion proteins. The purified proteins were characterised by analysis on SDS-PAGE followed by Coomassie staining and western blotting with a polyclonal rabbit anti-CLIC4 antiserum. Based on the identification of rCLIC4 binding partners in rat brain cytosol by affinity chromatography, followed by mass spectroscopic analysis and microsequencing, the interactions of rCLIC4 with α-tubulin, β-actin and 14-3-3ζ, were confirmed *in vivo* by immunoprecipitation in rat brain cytosol and in rCLIC4 stably transfected HEK-293 cells. However, as shown by subcellular fractionation experiments endogenous and recombinant CLIC4 occupy overlapping but distinct locations. Therefore the partners identified by recombinant CLIC4 may not necessarily be partners of endogenous CLIC4. rCLIC4 binding assays with immobilised GST 14-3-3ζ confirmed that their interaction is direct. In the presence of competitive Raf peptides (phosphorylated and non-phosphorylated form) the binding of rCLIC4 with 14-3-3ζ decreased . To further characterise rCLIC4 binding to β-actin, actin spin-down assays were performed. There was no evidence that rCLIC4 interacts with β-actin directly suggesting that rCLIC4 might bind to F-actin indirectly or that it may bind to non-polymerised G-actin. Finally rCLIC4 was shown to be *in vitro* phosphorylated by PKC, cAMP-dependant protein kinase A and casein kinase II, and *in vivo* phosphorylated by tyrosine kinase in HEK-293 cells.

CHAPTER 5

RECONSTITUTION OF rCLIC4 INTO PLANAR LIPID BILAYERS

5.1 INTRODUCTION

5.1.1 GENERAL PRINCIPLES OF ION CHANNEL RECONSTITUTION

Ion channel reconstitution involves the incorporation of membrane proteins into artificial phospholipid bilayers to permit the characterisation of channel function under voltage-clamp conditions. This procedure is based on the assumption that complex biological functions can be analysed by purifying the individual components and then recombining them in an appropriate manner. Ion channel reconstitution therefore makes possible the assessment of the properties of purified ion channel proteins and improves further the investigation of channels inaccessible to conventional electrophysiological approaches (for example intracellular channel proteins).

Two major steps are used to reconstitute ion permeability functions. The first involves the production of artificial phospholipid bilayers into which the channel protein can be incorporated. In general, the bilayer is formed across a hole that connects two fluid-filled chambers. This is achieved either by spreading phospholipid dispersion (painted bilayers; described in section 2.5.7.2) or by forming phospholipid monolayers at an air-water interface followed by folding into bilayers. The formation and folding of monolayers result in membranes that contain less solvent. The second step for reconstitution involves direct incorporation of the channel proteins into the artificial phospholipid bilayers. The channel proteins can be included in a native membrane vesicle (obtained for example by cell fractionation – homogenisation and centrifugation), or they can be isolated from the membrane following solubilisation with a detergent. These purified channel proteins may either

be in a solubilised form exposed to detergent, or have been inserted into proteoliposomes. Most methods for formation of proteoliposomes involve co solubilisation of the lipids and protein(s) in non-denaturing detergent solution followed by removal of the detergent by dialysis, dilution or gel filtration. In some cases proteins can be inserted into pre-formed lipid vesicles (liposomes) by freeze thaw or sonication.

Channel proteins in native membrane vesicles or in reconstituted proteoliposomes are probably inserted into phospholipid bilayers by fusion. Prior to fusion, vesicles are thought to associate with, or bind to the bilayer (pre-fusion state). The probability of this association occurring can be enhanced by a number of factors such as a net negative charge of phospholipids of either the membrane vesicle or the bilayer, addition of divalent or trivalent cations in the solution, stirring of the solution containing the vesicles, and increasing the surface area of the bilayer. The vesicle membrane will then fuse with the bilayer if they are induced to swell (fusion state). This is routinely achieved by the establishment of an osmotic gradient across the bilayer. Osmotic gradients generated with salts will induce fusion if the vesicle contains channel proteins permeable to either the cation or anion.

5.1.2 SINGLE CHANNEL ANALYSIS

The main function of an ion channel is to permit an electric current of ions to flow across a cell membrane in a passive manner. As described previously, single-channel currents can be recorded with patch clamping (as small as 0.1 pA) or with artificial lipid bilayers. These recordings provide two basic types of information, the

amplitude of the current and the dwell times. The current of a channel is measured as the difference of the open levels. The dwell time of a channel is the amount of time a channel remains in the closed state. Following the incorporation of channel protein into a lipid bilayer, the current flow through the channels is monitored using an amplifier. A detailed description of single-channel recordings and analysis is reviewed by Moss and Moczydlowski in Ion Channels: A practical approach (Chapter 3, Ashley 1995).

5.2 PREPARATION AND CHARACTERISATION OF SMALL UNILAMELLAR rCLIC4-LIPOSOMES

5.2.1 FORMATION OF rCLIC4-LIPOSOMES

In an attempt to resemble the membrane environment wherein rCLIC4 could generate channel activity the role of the protein to lipid ratio and of the specific lipids was examined by varying the lipid composition of the reconstituted mixture. Usually, proteoliposomes were prepared in 1:100, 1:500 and 1:1000 (w/w) protein to lipid ratio. In addition, two main lipid mixtures were used for the preparation of liposomes and proteoliposomes. The first mixture was from soybean lecithin (known also as asolectin). This is a very crude lipid mixture containing both charged phospholipids and neutral lipids. The second lipid mixture, previously described by Villar *et al.*, (1999), resembles the lipid environment of the caveolae/raft domains. The composition of this lipid mixture was: egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), cholesterol (Ch) and sphingomyelin (Sm) prepared in molar ratio 4:2:2:1. Finally the effects of detergents in vesicle reconstitution were

examined. Liposomes and proteoliposomes were prepared in the presence of 1.4 % (w/v) CHAPS, 1 % (w/v) octyglucoside or 1 % (w/v) cholate. The success of reconstitution using the detergents mentioned was examined by vesicle incorporation into lipid bilayers.

5.2.2 EVIDENCE OF SUCCESSFUL RECONSTITUTION INTO LIPID VESICLES

In order to characterise the reconstituted artificial membrane system, the liposomes containing rCLIC4 protein were subjected to a density gradient centrifugation on a sucrose gradient as described previously by Boffelli *et al.*, (1997). Visual inspection of the density gradient at the end of the centrifugation step showed a narrow turbid band at the interface between the 5 % and 10 % (w/v) sucrose layers (at the top of the quarter of the centrifuged tube). As shown in Figure 5-1, immunoblotting analysis of the sucrose gradient fractions with anti-rCLIC4 revealed that rCLIC4 was concentrated in fractions 2-4 (5 % - 10 % (w/v) sucrose interface). In the absence of phospholipids all the protein was found at the bottom of the gradient, while in the absence of protein all phospholipids were found at the 2.5 % – 5 % (w/v) sucrose interface.

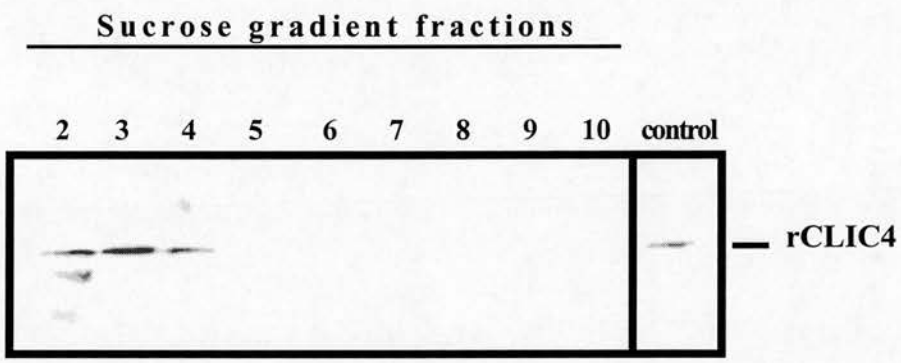


Figure 5-1. Sucrose gradient isolation of rCLIC4-liposomes.

Proteoliposomes (1 ml) were thoroughly mixed with 60 % (w/v) sucrose (1 ml) in 150 mM KCl, 5 mM Tris-HCl, pH 7.2, supplemented with 0.05 % (v/v) Triton X-100. The addition of Triton X-100 was necessary because proteoliposomes are impermeable to sucrose. Following successive layering with 20 %, 10 %, 5 % and 2.5 % (w/v) sucrose, the gradients were centrifuged (1 hr at 30,000 rpm in a Beckman SW41 rotor), collected in 1.0 ml fraction and analysed for protein content by immunoblotting with anti-CLIC4 immune serum. rCLIC4 was detected at the 5 % - 10 % (w/v) sucrose interface (fractions 2-4). Purified recombinant rCLIC4 was used as control.

5.2.3 ESTIMATION OF TRAPPED VOLUME

To further characterise the reconstituted vesicles the average vesicle's size was estimated. This is achieved by calculating the trapped (captured) volume and the encapsulation efficiency of the vesicles. The trapped volume depends on the radius of a specific liposome population (*i.e.* of a specific lipid composition) produced by a certain technique (for example detergent dialysis and gel filtration). There are several methods for determining the above parameters, either by direct measurement of the trapped compound or electron microscopic observation of individual vesicles. In this study a simple method was employed as described by Hope *et al.*, (1986). Liposomes were prepared in the presence of sodium chromate (10 mM final concentration), followed by detergent dialysis and gel filtration to remove the remaining external (non-trapped) chromate (section 2.5.6.1). The trapped chromate solution was determined by solubilising aliquots of the liposome in Triton X-100, followed by measurement of A_{380} for chromate. On average proteoliposomes had an encapsulation efficiency of 1 % which corresponds to ~ 200 nm vesicle diameter (as given by Hope *et al.*, 1986).

5.3 SINGLE CHANNEL RECORDINGS

To incorporate channels into the formed lipid bilayer, soluble rCLIC4 and rCLIC4-containing liposomes were stirred into the *cis* chamber. For control experiments lipids only liposomes without rCLIC4 were also prepared and added into the *cis* chamber. Fusion of liposomes and proteoliposomes with the lipid bilayer was further stimulated by the addition of divalent cations (Ca^{2+}) to *cis* chamber. Successful fusion was observed as spike-like, low amplitude (<1 pA) capacitative current transients, due to the increase of the lipid bilayer area following fusion. Table 5-1 summarises the solutions and the experimental conditions used for the channel activity recordings.

Analysis of the recordings for rCLIC4-liposomes incorporation revealed the presence of inconsistent and no well-defined "step-sized" unit currents. Out of a total of 44 experiments performed, 16 bilayers (37 %) ruptured within 30s of vesicle addition, usually while being stirred. In the remaining 28 attempts (63 %), no channel activity was observed in 22 (50 %) experiments even after 1 hr, whereas channel-like activity was observed in 6 (14 %) occasions only (anion channel activity 1 experiment (2 %), cation channel activity: 5 experiments (12 %)). In the experiments showing channel activity rCLIC4-liposomes were prepared with asolectin in 1.4 % w/v CHAPS, 20 mM Tris-HCl pH 7.4, P:L 1:100 w/w, 350:50 mM *cis* :*trans*. No channel activity was observed when lipids only liposomes were incorporated into the lipid bilayers.

To clarify whether the observed channel-like activity was anionic or cationic, the membrane potential was held at 0 mV and the direction of the deflections was observed, in the presence of a salt gradient (*cis* to *trans*). Anion channel activity was identified as "step-like" negative unit currents flowing from *cis* to *trans,* in the presence of a *cis* > *trans* concentration gradient. Under these conditions (0 mV) there was no electric driving force for anions (or cations), so that ions could only pass through the channels by flowing down their concentration gradient. On some occasions, cation-conducting channels were incorporated, usually observed when KCl was used to establish the salt gradient across the bilayer chambers (Figure 5-2). Upon successful incorporation, transmembrane currents were digitally recorded. To reduce high frequency noise the recordings were low-pass filtered at 50 Hz.

COMPONENT	REAGENT	RANGE
BUFFER	- MES-KOH	20 mM pH 6.0
	- Tris - HCl	20 mM pH 7.4 8.0 10.0
LIPID MIXTURE	- Crude lipid mixture	Soybean lecithin type IV (asolectin)
	- "Caveolae-like"	PC : PE : Ch : Sm 4:2:2:1 (molar ratio)
DETERGENT	- CHAPS	1.4 % (w/v)
	- n-Octyglucoside	1 % (w/v)
	- Na-Cholate	1 % (w/v)
PROTEIN	- Soluble rCLIC4	1 ng - 5ng
	- rCLIC4 -liposomes Protein : Lipid ratio (w/w)	1:100 1:500 1:1000
SALT GRADIENT (*cis* : *trans*)	- KCl	300 : 50 (mM) - 890 : 50 (mM)
	- CsCl	300 : 50 (mM) - 890 : 50 (mM)

Table 5-1. Single channel recordings: Summary of solutions and experimental conditions.

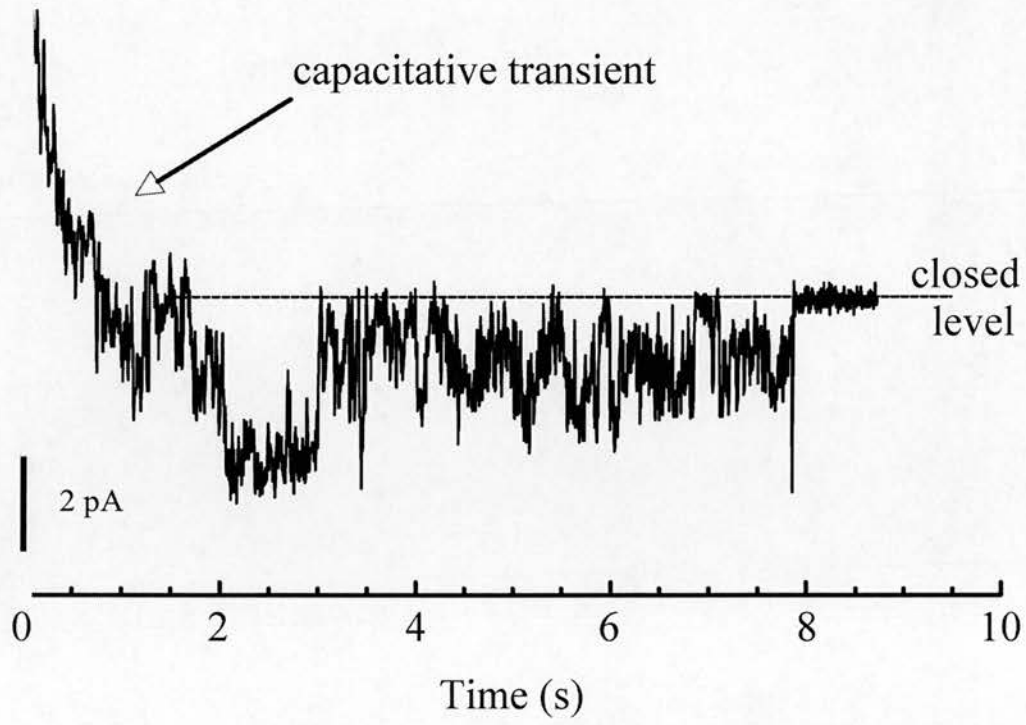


Figure 5-2. Channel activity recordings of rCLIC4-vesicles.

A single-channel recording at a holding potential switched from 0 to – 80 mV (capacitative transient) after incorporation of rCLIC4-containing liposomes (protein to lipid ratio 1:100) into a voltage-clamped lipid bilayer, in the presence of 350:50 mM KCl (*cis-trans*). The closed level is shown by the horizontal line. The downward deflections represent cation K^+ flux from *cis* to *trans*, low-pass-filtered at 50 Hz.

5.4 SCRAMBLASE ACTIVITY

The inconsistent and non-specific channel-like activity of rCLIC4 in lipid bilayers raised the hypothesis that rCLIC4 is a membrane active protein and it may mediate a Ca^{2+}-inducible phospholipid scramblase function. The phospholipid scramblase facilitates a rapid bi-directional movement of plasma membrane phospholipids across the bilayer. In preliminary experiments carried out by R.H Ashley, NBD-PC (1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol- 4 -yl) amino] caproyl - *sn* -glycero -3 -phospho-choline) labelled liposomes containing rCLIC4 were incubated at 37°C in the presence or absence of sodium dithionite which converts the NBD group to the non-fluorescent derivative. When the signal was stabilised, Ca^{2+} was added and the change in fluorescence was recorded. The signal observed was unaffected by the presence of rCLIC4-containing liposomes.

5.5 SUMMARY

The channel activity of rCLIC4 was extensively examined using the lipid bilayer set up. Purified recombinant rCLIC4 was mixed with lipids, and reconstituted into liposomal vesicles. The lipid mixture was chosen to resemble the natural environment where rCLIC4 may function as a channel protein. Following vesicle preparation, rCLIC4-containing liposomes were added to the *cis* chamber of the bilayer set up and allowed to fuse with the lipid bilayer. Analysis of the recorded channel activity revealed inconsistent and non-specific rCLIC4-associated channel activity.

CHAPTER 6

DISCUSSION AND CONCLUDING REMARKS

6.1 DISCUSSION

CLIC4, originally named p64H1 (Duncan *et al.*, 1997), was among the first members of putative intracellular anion channel proteins (known as $\underline{Cl}^-$ Intracellular Channel proteins, CLICs) to be identified at the molecular level. CLIC4 isoforms in different species (rat, human, mouse) are about 95% identical at the amino acid level, suggesting strong conservation through evolution.

In order to identify the function of rat brain CLIC4, a further investigation into its subcellular localisation was undertaken. Immunofluorescence staining of HEK-293 and MDCK cells transfected with Flag-tagged and non-tagged rCLIC4 showed that rCLIC4 has an unusual dual distribution in cytosolic and membranous fractions. This dual localisation of rCLIC4 was observed in both cell lines and it was further supported by western blot analysis of transfected HEK-293 cell lysates subjected to subcellular fractionation. rCLIC4 was present in both cytosolic (S3) and membrane (P2/3) fractions. These data combined are consistent with the characteristic, though unusual, localisation of many CLIC and p64-related proteins.

The localisation of recombinant rCLIC4 in sites near the plasma membrane raised the hypothesis that the protein exists within the membrane-specialised microdomains, commonly referred to as lipid rafts (Brown and London, 1998). These dynamic structures are rich in tightly packed sphingolipids and cholesterol, and concentrate a number of signal transduction molecules such as nitric oxide synthase, ligand gated receptors and protein kinases (Simons and Ikonen, 1997). Different types of rafts have been reported based on the presence of specific marker proteins and their ultrastrucure characteristics. Caveolae are probably the most well studied

subpopulation of lipid raft compartments (Parton, 1996). Previous studies of detergent-insoluble microdomains isolated from neurons, neuroblastoma cells and brain regions have not detected caveolin protein or caveolin mRNA (Lisanti *et al.*, 1994; Wu *et al.*, 1997; Shyng *et al.*, 1994). However, these regions contain typical caveolar glycolipids (Tettamanti and Riboni, 1993) and caveolin-like proteins (Lang *et al.*, 1998), which are mainly located in synaptic membranes.

To test the hypothesis that CLIC4 is involved in caveolar endocytosis, the association of rCLIC4 with endogenous caveolin, a marker of caveolae domains, was examined in HEK-293 and MDCK cells by indirect immunofluorescence. Dual immunostaining of HEK-293 cells expressing CLIC4 with anti-CLIC4 and anti-caveolin antibodies revealed only partial overlap of the two proteins. In MDCK cells, although both caveolin and CLIC4 display punctuate distribution, there is no co-localisation of the two proteins. This, however, is not unusual since the subcellular localisation of CLIC4 varies according to the cell type and species examined. In human pancreatic carcinoma cells, hCLIC4 co-localises with caveolae, consistent with our results, and *trans*-Golgi network (Edwards, 1999). In rat hippocampal neurons, rCLIC4 was associated with large dense core vesicles (Chuang *et al.*, 1999). mtCLIC4 has also been shown to localise in the mitochondrial and cytoplasmic compartments of mouse keratinocytes (Fernandez-Salas *et al.*, 1999).

Western blot analysis of sucrose-gradient fractions from HEK-293 cells stably expressing rCLIC4, demonstrated the distribution of rCLIC4 in isolated low density, Triton X-100 insoluble complexes (caveolae), as well as in the non-raft fractions. These results are consistent with the partial co-localisation of rCLIC4 with

endogenous caveolin observed by immunofluorescence. Caveolin failed to immunoprecipitate with anti-CLIC4 immune serum in cells suggesting that there is no physical interaction between these two proteins and that the partial co-localisation of rCLIC4 with caveolin is solely because rCLIC4 is present in caveolae domains or interacts with proteins present in these domains. To further determine whether rCLIC4 associates with functional caveolae, in experiments carried out by Dr. R.H. Ashley, rCLIC4-transfected HEK-293 cells were pre-incubated with FITC-labelled cholera toxin B subunit (CT B) prior to fixation. CT B subunit was readily taken up by HEK-293 cells and was partially co-localised with rCLIC4.

At least two more channel proteins have been reported to co-localise with lipid raft domains: the cardiac Na^+ channel (Yarbrough *et al.*, 2002) and the Shaker-like, voltage-gated K^+ (Kv) channel (Martens *et al.*, 2001). As it was previously suggested the channel/raft association could serve primarily to cluster signalling molecules (including protein kinases) with ion channels (Martens *et al.*, 2001). rCLIC4 protein has multiple phosphorylation sites and it was shown to be tyrosine phosphorylated in HEK-293 cells. Tyrosine kinases have been previously reported to be localised to lipid raft microdomains including those from neuronal plasma membrane (Wu *et al.*, 1997), supporting the functional localisation of CLIC4 to rafts. The mechanism of channel/raft association is yet unclear. The channel could bind the raft-associated proteins (including their markers *e.g.* caveolin) or it could directly target to or interact with the lipid raft. One strong candidate is dynamin, which was shown (by W. Suginta) to bind directly to rCLIC4 by gel overlay and reverse pull down assays.

Dynamin has been recently reported to mediate caveolae budding (internalisation) during the formation of transport vesicles (Henley *et al.*, 1998; Oh *et al.*, 1998). Dynamin is a mechanoenzyme that was originally identified as microtubule-binding protein (Paschal *et al.*, 1987; Shpetner and Vallee, 1989). It belongs to a large multigene family of high molecular weight (100 kDa) GTPases and has been shown to bind and hydrolyse GTP. Mammals have three distinct dynamin genes that are expressed in a tissue specific manner. Dynamin I is expressed exclusively in neurons (Obar *et al.*, 1990), dynamin II is found in all tissues (Cook *et al.*, 1994; Sontag *et al.*, 1994) and dynamin III is restricted to the testis, the brain, the lung and the heart (Cook *et al.*, 1996; Nakata *et al.*, 1993). Furthermore each dynamin gene encodes four or more alternatively spliced isoforms, which at least some of these variants have been shown to have further distinct cytoplasmic distributions (Urrutia *et al.*, 1997).

Several reports suggest that the importance of such diversity is related to the interactions of dynamin with a variety of soluble, cytoskeletal or membrane-associated proteins (including signalling molecules and molecules involved in the endocytic machinery) as well as lipids (McNiven *et al.*, 2000a). Considering the wide and similar tissue distribution of dynamin isoforms (and their spliced forms) and that of CLIC4 it is safe to suggest that CLIC4 could potentially interact with one or more spliced forms of dynamin II or dynamin III. It is well established that dynamin is involved in receptor-mediated endocytosis, synaptic vesicle recycling and possibly in vesicle transport from the endosomes to the Golgi (Hinshaw, 2000). In addition to the partial co-localisation of rCLIC4 with caveolae, the association of dynamin I with rCLIC4 further supports its involvement in synaptic vesicle endocytosis.

rCLIC4 was also shown to interact with the cytoskeletal proteins α-tubulin and β-actin. Following the association of CLIC4 with dynamin, the presence of α-tubulin in the CLIC4 protein complex is not a surprise. Indeed, as mentioned earlier, dynamin was originally characterised as a microtubule binding protein (Obar *et al.*, 1990). Furthermore, in neuronal plasma membranes tubulin has been reported to localise to the caveolae-like domains via a lipid anchor (Palestini *et al.*, 2000), consistent with the partial localisation of CLIC4 to caveolae domains in HEK-293 cells. The association of CLIC5, another member of the CLIC family, with several cytoskeletal proteins of placenta microvilli (actin, ezrin, α-actinin, gelsolin; (Berryman and Bretscher, 2000) is of great interest. However, the results of actin spin-down assays suggest that the interaction of rCLIC4 with actin is not direct. It is noticeable that the actin cytoskeleton acts synergistically with dynamin in the alteration of membrane shape and form during cell migration, endocytosis and secretion (Lee and De Camilli, 2002; Orth *et al.*, 2002). The interactions of the actin binding proteins, profilin (Witke *et al.*, 1998) and cortactin (McNiven *et al.*, 2000b), with dynamin provide strong evidence for a functional implication of actin in endocytosis as well as in caveolae-mediated endocytosis (Pelkmans *et al.*, 2002). These findings are consistent with the presence of actin in the rCLIC4 / dynamin protein complex and CLIC4 involvement in caveolar-mediated endocytosis.

Finally rCLIC4 was shown to associate with 14-3-3 proteins. 14-3-3 proteins comprise a family of highly conserved acidic proteins, distributed in almost all tissues and especially in brain where they account for approximately 1 % of its total soluble proteins (Boston *et al.*, 1982). Several functions have been attributed to the 14-3-3 proteins both at the cellular level (stimulation of exocytosis and regulation of

cell cycle; (Morgan and Burgoyne, 1992; Ford *et al.*, 1994)) and at the molecular level (regulation of protein kinase C and the tyrosine and tryptophan hydroxylases; (Isobe *et al.*, 1992; Yamauchi *et al.*, 1981). Interestingly, 14-3-3 proteins were implicated in activation of Ca^{2+}-dependent Cl^- channels (CaCC) in *Xenopus* oocytes suggesting a potential role of 14-3-3 proteins in the regulation of epithelial Cl^- secretion (Chan *et al.*, 2000). However the functional mechanism of the 14-3-3 proteins is unclear. In addition to a possible role of the 14-3-3/CLIC4 association in the regulation of epithelial Cl^- secretion, 14-3-3 proteins bound directly to the brain CLIC4 protein complex, could mediate protein kinase C–dependent CLIC4 phosphorylation or they may function as adapter proteins by coupling CLIC4 to other signalling molecules.

Protein phosphorylation and dephosphorylation are among the most important protein modifications. Phosphorylation and dephosphorylation are carried out by protein kinases and phosphatases respectively on serine, threonine and tyrosine residues and result in the activation or inhibition of the protein. Phosphorylation of tyrosine residues can also create highly selective docking sites for SH2 or other phosphotyrosine binding-domain containing proteins.

In this study rCLIC4 was found to be phosphorylated *in vitro* by PKC (reported previously by (Duncan *et al.*, 1997), PKA and casein kinase. rCLIC4 was also shown to be tyrosine phosphorylated in HEK-293 cells expressing rCLIC4 when treated with tyrosine phosphatase inhibitors prior to immunoprecipitation.

As described previously, other members of the CLIC/p64 super-family are phosphorylated by, and/or associate with protein kinases. Similar to CLIC4, p64, the founder member of this super-family contains consensus phosphorylation sites for PKA, PKC, casein kinase II and tyrosine kinase lying within consensus sequences for binding by various SH2 domain proteins (Edwards *et al.*, 1998; Edwards and Kapadia, 2000). In HeLa cells, p64 becomes tyrosine phosphorylated when co-expressed with $p59^{fyn}$, a non-receptor tyrosine kinase member of the Src family, resulting in enhanced p64-associated Cl^- channel activity (Edwards and Kapadia, 2000). Furthermore, a fraction of native kidney p64 interacts *in vitro* with the Fyn-SH2 domain. However, is not clear whether tyrosine phosphorylation and/or subsequent binding of the phosphorylated p64 to the Fyn-SH2 domain could alter activity and targeting of the protein. Another member of the CLIC family, CLIC3 was reported to associate with ERK7, a member of the mitogen-activated protein kinase family, but not to be phosphorylated by this kinase (Qian *et al.*, 1999). Although the functional role of the interaction between CLIC3 and ERK7 is not yet clear, it was suggested that ERK7 might mediate phosphorylation of CLIC3 and/or chloride channel conductance. Finally CLIC1 was found to associate with AKAP350, a member of the A kinase anchoring proteins (AKAPs) which bind the regulatory subunit of type II protein kinase A (Schmidt *et al.*, 2000). In addition, co-expression of AKAP350 and CLIC1 in HCA-7 cells led to redistribution of both proteins in the Golgi apparatus. The functional consequences of this interaction are still unclear.

The single channel behaviour of rCLIC4 was extensively examined in planar lipid bilayers. Solubilised recombinant rCLIC4 or rCLIC4 -containing proteoliposomes were incorporated into lipid bilayers and the channel activity was recorded. The observed channel activity was inconsistent and mostly associated with cation selective channels rather with anion selective channel. These results suggest that rCLIC4 does not function as channel protein. However, Duncan *et al.*, (1997) showed that incorporation of microsomal membrane fractions from HEK-293 cells expressing rCLIC4 (p64H1) into lipid bilayers, gave rise to novel intracellular anion channel activity. It is possible that rCLIC4 is associated with other subunits (present in cells but absent in bacteria), which might modify and regulate rCLIC4 channel activity. However, no such subunits were detected in the protein-protein interaction studies that could trigger Cl^- channel activity. Another possible explanation is that expression of rCLIC4 in cells could initiate (either by phosphorylation or association with other proteins such as dynamin) a cascade of events, which further result in the activation of endogenous channels distinct from rCLIC4.

The best electrophysiologically characterised members of the CLIC/p64 super-family are p64 and CLIC1 (NCC27). Unlike CLIC1, the evidence presented to ascribe a channel protein role to p64 is indirect (Landry *et al.*, 1989; Redhead *et al.*, 1992; Landry *et al.*, 1993; Edwards *et al.*, 1998). These include co purification of p64 with channel activity and immunodepletion of Cl^- channel activity from solubilised bovine kidney microsomal membranes. Single channel data recorded from fusion of purified p64 with planar lipid bilayers, showed the presence of three distinct anion channels with different conductance properties (Landry *et al.*, 1989). The authors however did not address the possibility that p64 might regulate/activate

other channel(s) which may have been co purified with p64 or why its activity is not inhibited by IAA compounds.

Reconstitution of recombinant, soluble CLIC1 expressed in bacteria into phospholipid membranes in a pH-dependent process (Warton *et al.*, 2002), yields a chloride channel whose activity is inhibited by IAA (Tulk *et al.*, 2000; Warton *et al.*, 2002). Expression of CLIC1 in cultured cells has also been reported to associate with increased chloride channel activity in plasma and nuclear membranes (Tonini *et al.*, 2000; Valenzuela *et al.*, 1997). Interestingly, electrophysiological studies in Chinese hamster ovary (CHO-K1) cells showed that CLIC1 chloride conductance varied according to the stage of the cell cycle, and that it was expressed only on the plasma membrane of cells in G2/M phase (Valenzuela *et al.*, 2000). This strongly suggests a role of CLIC1 in cell cycle regulation. However, the channel properties of CLIC1 associated-channels vary between different studies (*e.g.* chloride-dependant conductance of 30 pS in 140 mM KCl, IC_{50} for IAA94 of 25 μm (Warton *et al.*, 2002), *vs* chloride-dependant conductance of 67.5 pS in 150 mM KCl, IC_{50} for IAA94 of 86 μm (Tulk *et al.*, 2000). One explanation of this variation may be the different experimental conditions under which the channel activity was characterised in each study.

CLIC1 is an unusual ion channel. It is a small protein with one putative transmembrane domain that mainly exists in a soluble form, with only small amounts of the protein being found in membranes. The X-ray crystal structure of its soluble form does not resemble that of an ion channel (Harrop *et al.*, 2001). Soluble CLIC1 is a monomeric protein, structurally homologous to the GST super-family and has a

redox active site resembling glutaredoxin. Preliminary experiments of soluble hCLIC1 and rCLIC4 revealed that none of these proteins have a GST-like enzymatic activity.

Based on the high-resolution crystal structure of the soluble CLIC1 form, rCLIC4 can be convincingly modelled using SWISS-MODEL (Peitsch *et al.*, 2000; http://www.expasy.org/swissmod/SWISS-MODEL.html). Similar to CLIC1 structure, CLIC4 model consists of a four-stranded mixed β-sheet (s1, s2, s3 and s4) plus ten α-helices (h1, h2 and h3, h4a, h4b, h5, h6, h7, h8 and h9), as shown in Figure 6-1. Figure 6-2 shows a stereogram of CLIC4 model where the potential protein interactions and phosphorylation motifs are highlighted. It is worth noticing the long loop between h5 and h6 at the *N* terminal domain. As reported previously, this loop is a distinctive feature of CLICs and it is characterised by a high negative charge (a net negative charge of 6 for CLIC4; (Harrop *et al.*, 2001). This region could be a very important in protein-protein interactions.

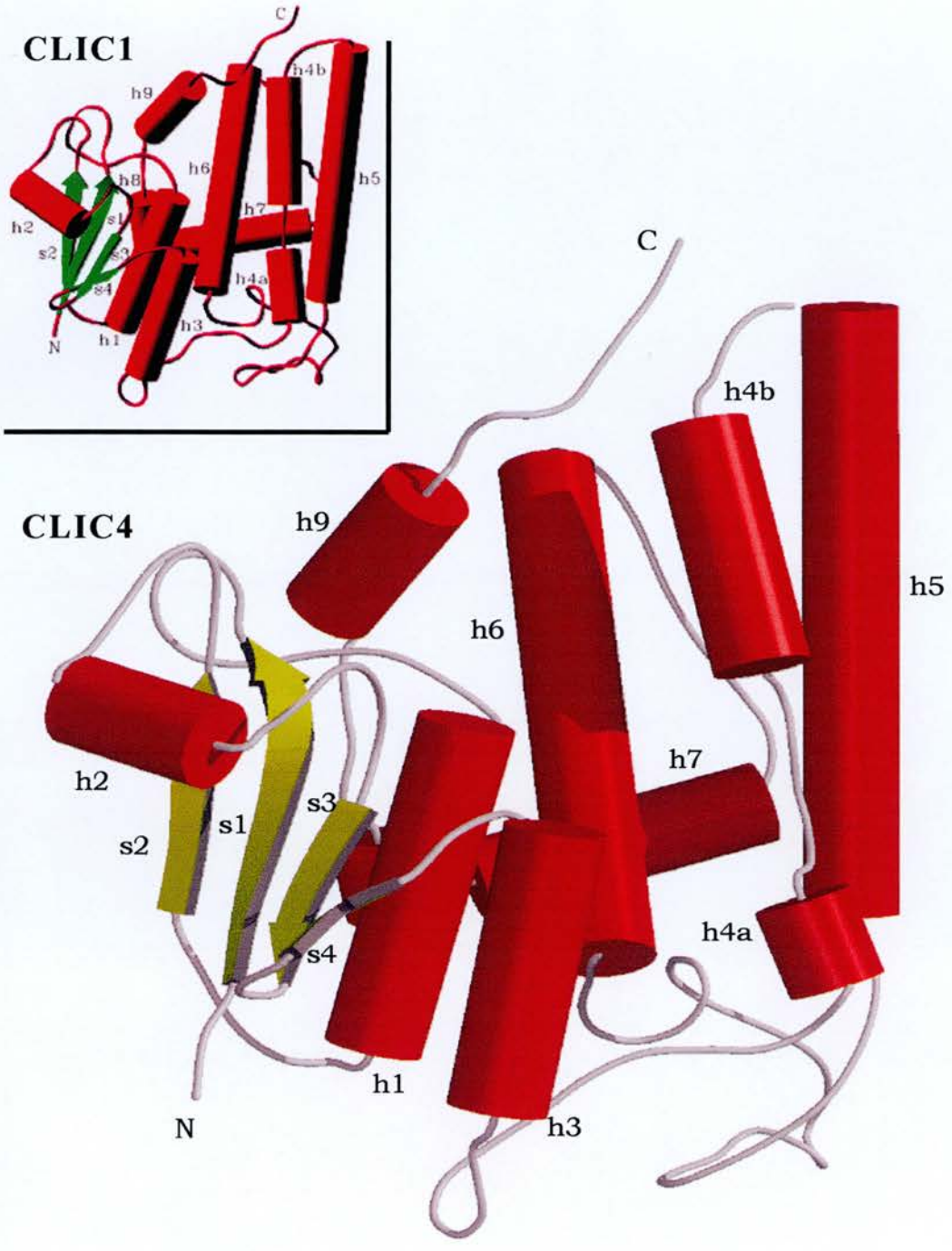
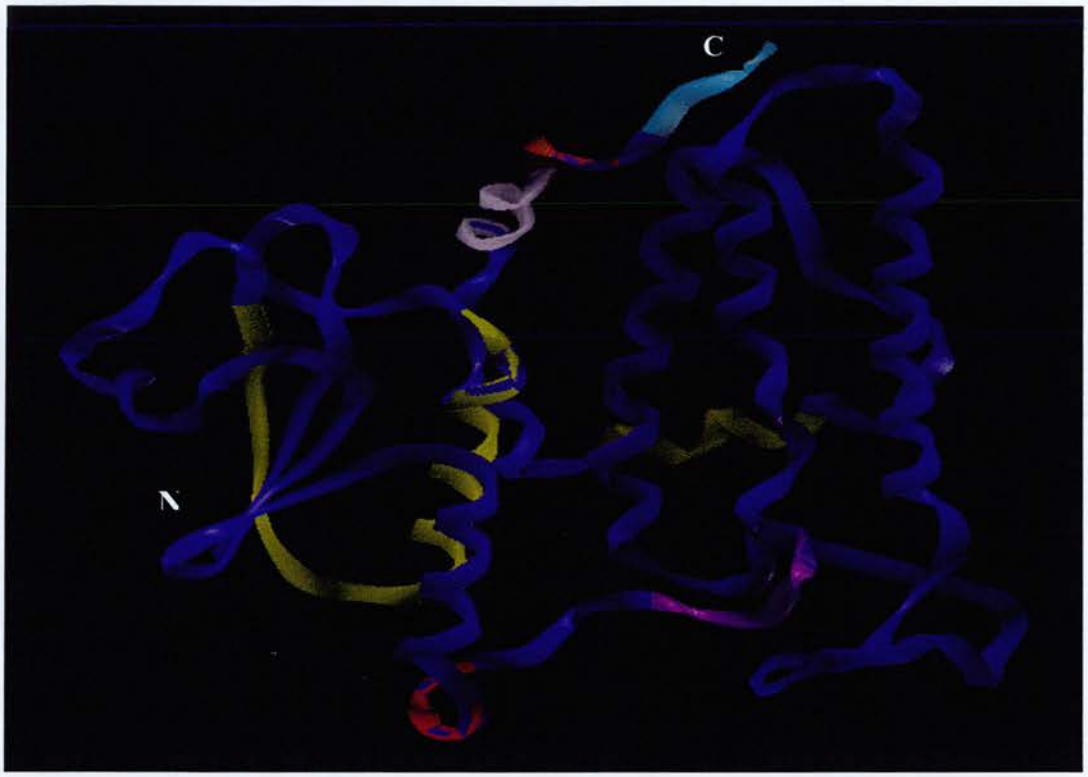


Figure 6-1. Homology modelling of rCLIC4.

CLIC4 model was generated using SWISS-MODEL (Peitsch *et al.*, 2000; http://www.expasy.org/swissmod/SWISS-MODEL.html), an automated Protein Modelling Server based on the atomic co ordinates of human CLIC1 (Protein Data Bank entry: 1K0M / Chain B) as the template (Harrop *et al.*, 2001). CLIC4 and

Figure 6.1 (continued)

CLIC1 (1K0MB) share a sequence identity of 64 % (SWISS-MODEL BLASTP2 output) with relative mean square deviations (rmsd) for the C_α atoms between the target and template structures being 0.08 Å (ProFit calculation [Martin ACR, http://www.bioinf.org.uk/software/profit]). Similar to CLIC1 structure, CLIC4 model consists of a four-stranded mixed β-sheet (s1, s2, s3 and s4) plus ten α-helices (h1, h2 and h3, h4a, h4b, h5, h6, h7, h8 and h9).



MALSMPLNGLKEEDKEPLIELFVKAGSDGESIGNCPFSQRLFMILWLKGVVFSVT
TVDLKRKPAHLQNLAPGTHPPFITFNSEVKTDVNKIEEFLEEVLCPPKYLKLSPK
HPESNTAGMDIFAKFSAYIKNSRPEANEALERGLLKTLQKLDEYLNSPLPGEIDE
NSMEDIKSSTRRFLDGDEMTLADCNLLPKLHIVKVVAKKYRNFDIPKGMTGIWR
YLTNAYSRDEFTNTCPSDKEVEIAYSDVAKRLTK

Figure 6-2. CLIC4 putative protein-interaction motifs.

Schematic representation of the putative protein-interaction motifs on CLIC4 model **(TOP)**. Complete amino acid sequence of CLIC4, from *N* to *C* terminus **(BOTTOM)**. Keys to highlighted amino acid sequences: predicted transmembrane domain (yellow), potential binding motifs for WW-domain (red) and SH3-domain (violet) proteins, 14-3-3 binding motif (light green), consensus phosphorylation sites for tyrosine kinase (white) and cAMP-dependant protein kinase (cyan), potential phosphorylated Ser and Thr residues (underlined) by PKC and CKII respectively. The YSDV motif (orange) could be potentially recognised by SH2-domain proteins.

6.2 CONCLUDING REMARKS AND FUTURE RESEARCH GOALS

The function of rCLIC4 is not yet understood. However, the experiments presented here demonstrated the association of soluble rCLIC4 with other cellular proteins supporting the idea that rCLIC4 may be a channel regulator. Further investigation of the interaction of rCLIC4 with dynamin could provide evidence for a possible role of soluble rCLIC4 in endocytosis. However, the channel activity of rCLIC4 remains in question. It is possible that the association of rCLIC4 with other cellular proteins acts as a targeting mechanism for rCLIC4 to membrane domains, where it functions as an ion channel. Another possibility is that the protein may be membrane active and that the inconsistent channel-like activity, observed during the reconstitution experiments in lipid bilayers, is the result of rCLIC4 auto-insertion into membranes. Currently the membrane insertion properties of rCLIC4 are being investigated using a monolayer setup. Preliminary results from these experiments indicate that the protein becomes membrane active upon treatment with oxidising reagents (*e.g.* H_2O_2), whereas treatment of rCLIC4 with reducing reagents (*e.g.* DTT) diminishes its membrane activity.

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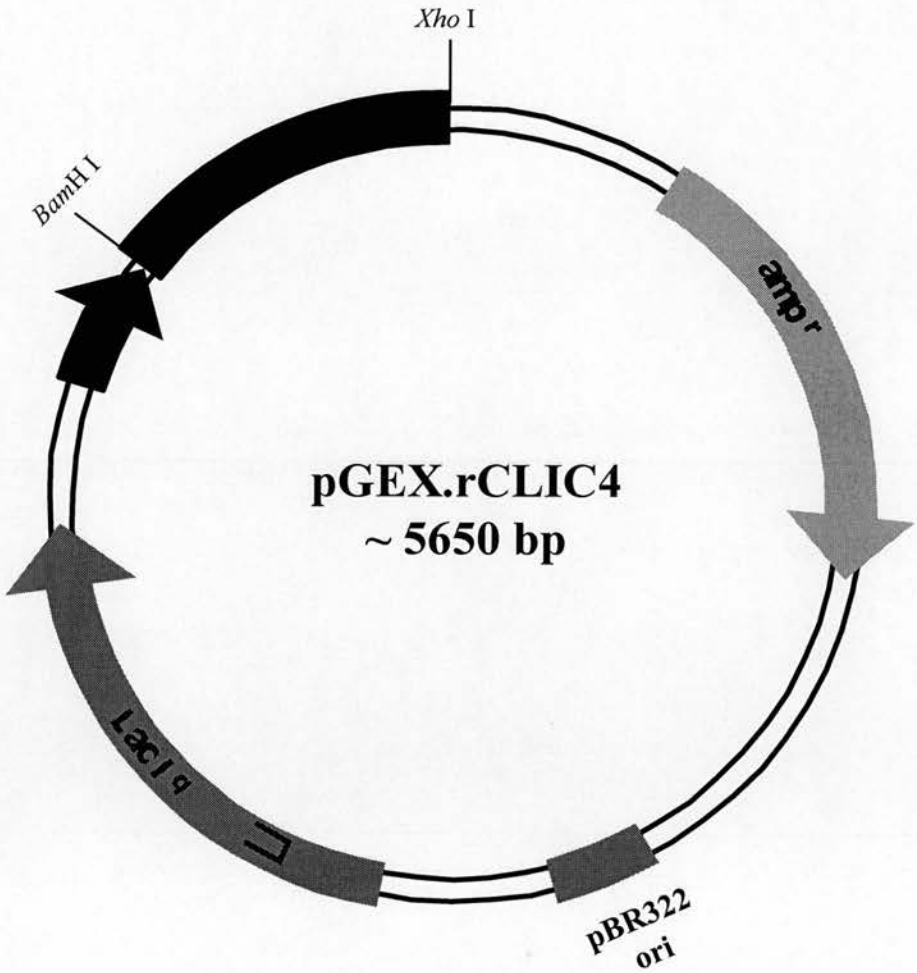
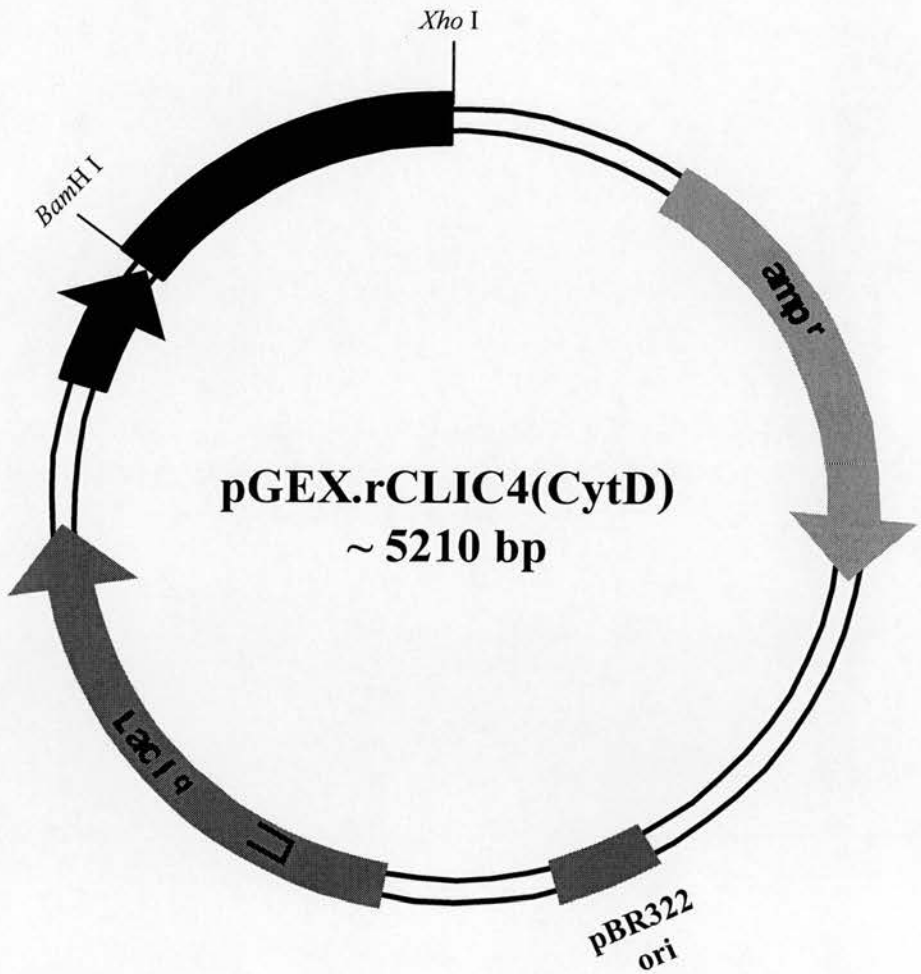
APPENDICES



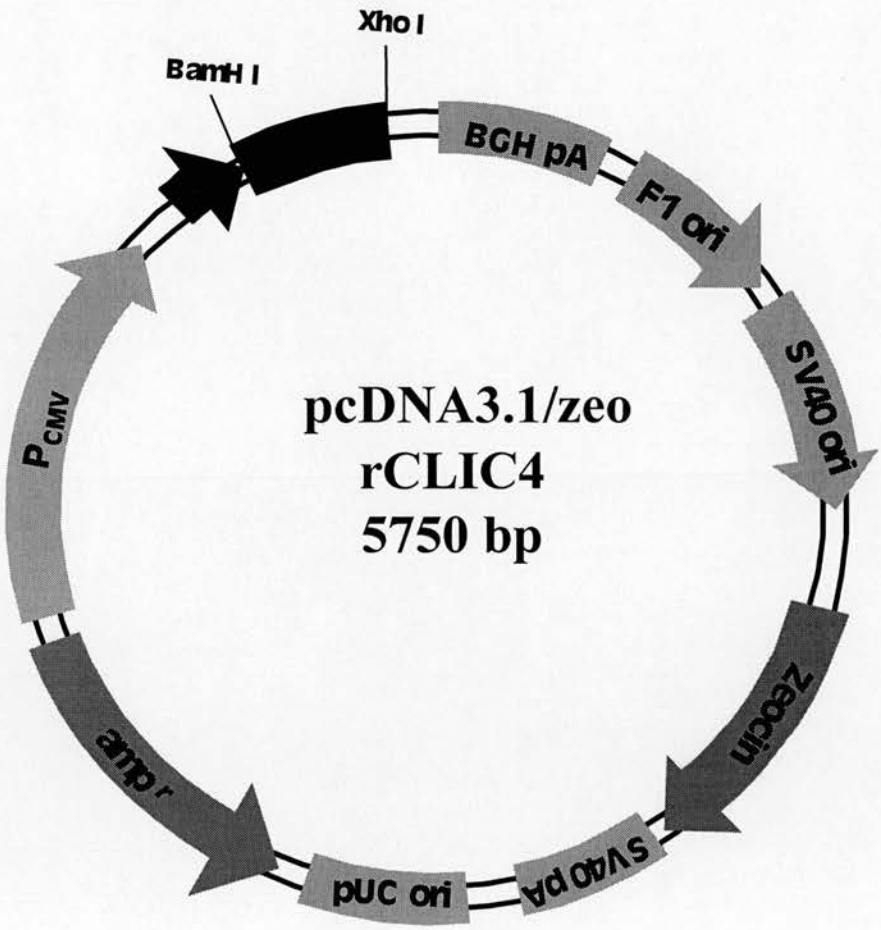
Plasmid pGEX. rCLIC4.

This plasmid contains a *Bam*H I – *Xho* I restriction fragment, encoding the full length rCLIC4 fused 'in frame' with the *C* terminus of GST sequence. The plasmid was used in the purification of rCLIC4 for the protein-protein interaction studies, phosphorylation assays and its reconstitution in lipid bilayers.



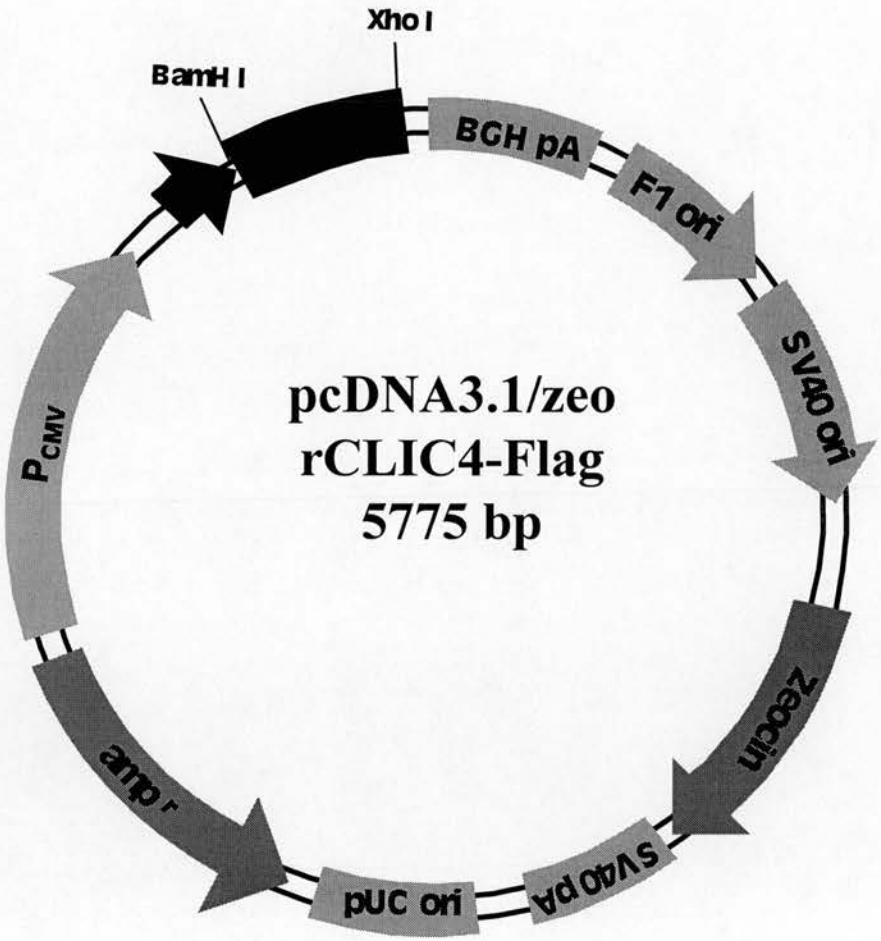
Plasmid pGEX. rCLIC4(Cyt.D.).

This plasmid contains a PCR-generated truncation of rCLIC4, encoding a protein lacking the *N* terminal and transmembrane domains. The plasmid was used in the purification of the rCLIC4 (Cyt.D.) for the protein-protein interaction studies.



Plasmid pcDNA3.1/zeo rCLIC4.

This plasmid contains a *Bam*H I – *Xho* I restriction fragment subcloned from pGEX4T1.rCLIC4. The restriction fragment contains the entire coding region of rCLIC4. The transcription of the insert is under the control of the CMV enhancer/promotor element. The plasmid was used in the transfection of HEK-293 and MDCK mammalian cell lines.



Plasmid pcDNA3.1/zeo rCLIC4-Flag.

This plasmid contains a *Bam*H I – *Xho* I restriction fragment, encoding rCLIC4 protein fused 'in frame' with the Flag epitope sequence. The plasmid was used in the transfection of HEK-293 and MDCK mammalian cell lines.

APPENDIX II: PUBLICATIONS

Suginta, W., Karoulias, N., Aitken, A., and Ashley, R.H. (2001). Chloride intracellular channel protein CLIC4 (p64H1) binds directly to brain dynamin I in a complex containing actin, tubulin and 14-3-3 isoforms. *Biochem J.* 359:55-64.